THE TOPOLOGY, REGULATION, AND FUNCTION OF CARDIOLIPIN REMODELING IN *SACCHAROMYCES CEREVISIAE*

by

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ABSTRACT

Cardiolipin (CL) is a unique phospholipid that resides exclusively within mitochondria, and is required for the optimal function of numerous mitochondrial processes. After synthesis, CL undergoes an evolutionarily conserved acyl chain remodeling process where acyl chains are removed by a lipase to form monolyso-CL (MLCL), and replaced by the transacylase tafazzin (Taz1p), reforming CL and establishing a final molecular form that is distinct from newly synthesized CL and highly homogeneous in an organism or cell type. Additionally, mutations in *TAZ1* cause Barth syndrome, resulting in cardiac and skeletal myopathy, cyclic neutropenia, and respiratory chain dysfunction.

The topology of CL biology is complex; synthesis occurs on the matrix-facing leaflet of the mitochondrial inner membrane (IM), but remodeling is completed on the intermembrane space-facing leaflets of both the IM and outer membrane (OM). We found that Cld1p, the lipase that initiates CL remodeling, functions between Crd1p and Taz1p, and resides in the matrix-facing leaflet of the IM but does not traverse the membrane. Thus, after MLCL is generated by Cld1p, it must translocate either across the IM or to the OM, identifying a previously unappreciated trafficking step required for CL remodeling.

Similar to other cellular processes which exhibit spatial separation of enzymatic steps in a pathway, the translocation of MLCL potentially represents a regulation point within CL remodeling. We found that the activity of MLCL translocation was not regulated in any condition we tested. Instead, Cld1p regulates CL remodeling. Its activity is modulated by the available carbon source or by changes in the mitochondrial

membrane potential; two separate mechanisms which allow CL remodeling to be regulated either coordinately with or independently from CL biosynthesis.

By analyzing Δ*cld1* yeast containing only unremodeled CL, we analyzed the functional differences between unremodeled and remodeled CL. We found that unremodeled and remodeled CL is equally able to support oxidative phosphorylation assembly and function, and mitochondrial morphology. This is in contrast to the widely accepted hypothesis that CL remodeling establishes a molecular form of CL optimized to support oxidative phosphorylation. Thus CL remodeling may have other physiological roles besides the establishment of a specific molecular form.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

Introduction

Sections of this chapter are derived from:

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MITOCHONDRIAL BIOLOGY

While the mitochondrion is best known for its ability to synthesize ATP through oxidative phosphorylation (OXPHOS), it additionally participates in a multitude of other fundamental cellular processes, including ion regulation, heme and Fe/S cluster synthesis, lipid metabolism, signal transduction, and programmed cell death. Because of its critical and pivotal roles in cellular physiology, mitochondria (or an organelle with mitochondrial-like activities) are essential in all eukaryotic cells.

The Mitochondrial Genome

Mitochondrial functions require the coordination of both the nuclear and mitochondrial genomes. Of the estimated 1000-1500 mitochondrial proteins in humans (approximately half of which are ubiquitously expressed in all tissues; Calvo and Mootha, 2010), only 13 are encoded by the mitochondrial genome (mtDNA), all of which are subunits of the respiratory chain. In addition, 2 rRNAs and all 22 tRNAs are encoded by mtDNA. Mitochondria in *Saccharomyces cerevisiae* (herein referred to as "yeast") contain roughly 1000 different proteins per mitochondrion (Reinders et al., 2006; Sickmann et al., 2003). Except for respiratory complex I subunits, which are absent in yeast, and Var1p, a constituent of the small ribosomal subunit, which is present in yeast (Terpstra and Butow, 1979), all mtDNA genes are conserved between yeast and humans.

Mitochondrial Protein Import

Since only ~1% of the mitochondrial proteome is encoded by mtDNA, most mitochondrial proteins must be targeted to mitochondria and sorted to the correct submitochondrial compartment (for a comprehensive review, see Chacinska et al., 2009).

After synthesis on cytosolic ribosomes, virtually all preproteins are recognized by the Translocase of the Outer Membrane (TOM) complex, which facilitates movement across the outer membrane (OM). OM β-barrel proteins are inserted by the Sorting and Assembly Machinery (SAM) complex, while proteins with α-helical transmembrane domains require Mim1p for their insertion into the membrane (Becker et al., 2011; Papic et al., 2011).

Insertion into the inner membrane (IM) or translocation into the matrix invariantly requires the proton-motive force generated by the electron transport chain. Polytopic IM proteins with internal sorting signals are inserted into the IM by the Translocase of the Inner Membrane (TIM) 22 complex. Notably, external ATP is not needed for this process. Substrates of the TIM22 translocon include members of the mitochondrial carrier family as well as Tim23p and Tim22p of the TIM23 and TIM22 translocons, respectively.

The TIM23 complex utilizes the mitochondrial membrane potential +/- ATP to deliver precursors containing N-terminal targeting signals to three distinct compartments: the matrix, the IM, and the intermembrane space (IMS). Import into the matrix is distinguished from release into the IM or IMS by additionally requiring ATP to fuel the Presequence translocase-Associated Motor, or PAM complex. For those precursors that contain a hydrophobic "stop-transfer" signal, the N-terminal targeting sequence is removed by the matrix processing peptidase followed by either the lateral release into the IM or, subsequent to further processing by additional proteases that remove the "stop-transfer" signal, release into the IMS.

Another pathway for the import of soluble IMS resident proteins is the disulfide relay system (reviewed in Chacinska et al., 2009; Endo et al., 2010; Hell, 2008; Koehler and Tienson, 2009; Riemer et al., 2011). Substrates of the disulfide relay system are characterized by their small size, lack of N-terminal targeting signal, and conserved cysteine residues. The cysteine residues are required for disulfide bond formation between the substrate and import machinery, and for proper protein folding. The IMS-resident receptor, oxidized Mia40p, forms a disulfide intermediate with incoming preproteins. Protein substrates are then released from Mia40p, leaving it in a reduced state. Erv1p, a FAD-dependent sulfhydryl oxidase, re-oxidizes Mia40p, allowing another substrate to bind. Finally, Erv1p reduces cytochrome *c* allowing the re-oxidized Erv1p to participate in another round of import. Thus, this pathway mediates the proper folding of soluble proteins that contain disulfide bonds, indicating that the IMS is not contiguous with the cytosol with regards to its redox chemistry as previously thought. The proper folding of substrates of this pathway is postulated to prevent their retrograde translocation through the TOM complex back into the cytosol via a folding trap mechanism. Lastly, it is worth mentioning that reduced cytochrome *c* donates its electron to complex IV and finally O_2. As such, the disulfide relay system contributes, albeit to a minor extent, to the ultimate generation of the membrane potential across the IM.

Oxidative Phosphorylation

Mitochondria generate the majority of a cell's energy via OXPHOS. The electron transport chain, consisting of respiratory complexes I-IV and the mobile electron carriers ubiquinone (coenzyme Q, CoQ) and cytochrome *c*, oxidizes reducing equivalents and pumps protons from the matrix, across the IM, to the IMS. The electrochemical gradient

that is generated is then utilized by the F_1F_o ATP synthase (complex V) to condense ADP and P_i to ATP.

Respiratory complexes are multisubunit assemblies which, except for Complex II, incorporate proteins encoded by both the mitochondrial and nuclear genomes. Thus the biogenesis of the OXPHOS system is a complicated and highly regulated process that involves chaperones and assembly factors that facilitate complex assembly as well as the insertion of cofactors (FAD, Fe/S, hemes, and Cu) required for electron transport (reviewed in Fernandez-Vizarra et al., 2009).

The NADH ubiquinone oxidoreductase (complex I) oxidizes NADH, transferring electrons to CoQ. It contains seven subunits encoded by mtDNA, with the remaining ~38 subunits encoded in the nucleus. Oxidation is performed by a flavin-mononucleotide moiety, and most of the Fe/S clusters (eight total) form a chain allowing electrons to flow to CoQ (Carroll et al., 2006; Efremov et al., 2010; Hunte et al., 2010; Koopman et al., 2010).

Due in part to its large size and its absence in the genetically-tractable yeast model, the assembly of complex I remains relatively ill-defined. Complex I likely undergoes modular assembly, but the specific steps and identities of many assembly factors remain enigmatic (Calvo et al., 2010; Ghezzi and Zeviani, 2012).

Yeast do not contain a complex I homolog. Instead, they utilize three NADH dehydrogenases to oxidize NADH and transfer electrons to CoQ (Luttik et al., 1998; Marres et al., 1991).

Succinate dehydrogenase (SDH, complex II) is simultaneously involved in two metabolic processes: in the TCA cycle, it oxidizes succinate to fumarate; and in the

electron transport chain, it transfers electrons from succinate to CoQ. SDH is composed of 4 nuclear encoded subunits, Sdh1-4 in yeast and SDHA-D in mammals. SDHA/Sdh1p is a flavoprotein that, with the Fe/S protein SDHB/Sdh2p, forms the catalytic core of SDH. SDHC/Sdh3p and SDHD/Sdh4p anchor the catalytic core to the matrix side of the IM and are the site of CoQ reduction (reviewed in Lemire and Oyedotun, 2002; Sun et al., 2005).

Two SDH assembly factors have recently been identified. Sdh5p (SDHAF2 in mammals) was identified in yeast as a soluble required for flavination of Sdh1p (Hao et al., 2009). The role of SDHAF1 remains to be defined, but patients with mutations in the gene present with infantile leukoencephalopathy (Brockmann et al., 2002; Bugiani et al., 2006; Ghezzi et al., 2009), and deletion of the yeast ortholog decreases SDH activity by 30-40% (Ghezzi et al., 2009).

The cytochrome *bc1* complex (complex III) transfers electrons from CoQ to cytochrome *c*. In mammals, complex III consists of 11 subunits, whereas in yeast complex III only contains 10 subunits. Three catalytic subunits are responsible for electron transfer: cytochrome *b* (Cobp; the only subunit encoded in the mitochondrial genome), cytochrome c_1 (Cyt1p), and the Rieske Fe/S protein (Rip1p) (Hunte et al., 2000; Iwata et al., 1998).

Only one complex III assembly factor has been identified, Bcs1p (Nobrega et al., 1992). It is an AAA protein that resides in the IM and facilitates the incorporation of Rip1p (Cruciat et al., 1999).

Cytochrome *c* oxidase (COX or complex IV) transfers electrons from cytochrome *c* to O_2. It consists of 3 mitochondrially encoded subunits, Cox1p, Cox2p, and Cox3p.

Cox1p and Cox2p contain the heme groups (*a* and a_3) and copper centers (Cu_A Cu_B) required for electron transfer. Cox3p may provide a structural role in stabilizing the catalytic core, or play a role in proton pumping (reviewed in Fontanesi et al., 2006). The additional subunits (10 in mammals, 8 in yeast) stabilize the catalytic core, but are nonetheless essential for COX function, since the disruption of most subunits abolishes COX assembly (Barrientos et al., 2002b).

Over 20 additional proteins are required for the proper assembly of COX in yeast (Barrientos et al., 2002a; Barrientos et al., 2009; Fontanesi et al., 2006; Petruzzella et al., 1998). Mss51p enhances Cox1p translation (Siep et al., 2000); and together with Cox14p and Coa3p, binds and stabilizes unassembled Cox1p (Barrientos et al., 2002b; Mick et al., 2010; Perez-Martinez et al., 2003). Worth mentioning, Mss51p in complex with unfolded Cox1p is unable to further stimulate Cox1p translation, reducing Cox1p production in the absence of productive COX assembly, Mss51p is titrated out resulting in a drop in Cox1p production. Coa1p acts to release Mss51p and Cox14p (Mick et al., 2007; Pierrel et al., 2007). Cox11p and Shy1p which are required for the addition of the Cu_B cofactor to Cox1p; and Coa2p, Cox10p, and Cox15p generate the heme *a* that is uniquely utilized by COX (Barros et al., 2001; Bestwick et al., 2010; Hiser et al., 2000; Khalimonchuk et al., 2010). Cofactor loaded Cox1p is further stabilized by two nuclear encoded subunits, Cox6p and Cox5p (Pierrel et al., 2008). The addition of Cu_A to Cox2p involves the Cu chaperone Cox17p, which traffics between the cytosol and IMS, and the copper binding protein Sco1p (and SCO2 in humans) (Beers et al., 1997; Glerum et al., 1996a; Glerum et al., 1996b). Steps downstream of this are at present ill-defined as are the exact mechanisms for the attachment of the numerous COX cofactors.

The mitochondrial F_1F_o ATP synthase (complex V) utilizes the proton gradient generated by the other respiratory complexes to condense ATP from ADP and P_i. Complex V consists of two functional units. F_1 faces the matrix and is hydrophilic. It consists of 5 subunits (3 α, 3 β, γ, δ, and ε), and is the section that generates ATP (or hydrolyzes it when not attached to the F_o). F_o is hydrophobic and embedded in the IM. It consists of 8 different subunits and mediates proton translocation through the oligomeric ring of c subunits (Atp9p in yeast). Only three subunits are encoded by the mitochondrial genome, Atp6p, Atp8p, and Atp9p, all of which are components of the F_o (Velours and Arselin, 2000).

Of the ATP synthase assembly factors identified in yeast (Ackerman and Tzagoloff, 1990; Rak et al., 2009), only Atp11p and Atp12p have functional homologs identified in humans (Wang et al., 2001). Atp11p and Atp12p bind selectively to newly imported β and α subunits of F_1, respectively (Wang et al., 2000), acting as chaperones to facilitate the assembly of the α/β hexamer of F_1.

Although the OXPHOS complexes have long been modeled to function in isolation, it recently has been appreciated that respiratory complexes form large, macromolecular structures termed “respiratory supercomplexes” (Cruciat et al., 2000; Schagger and Pfeiffer, 2000). Respiratory supercomplexes increase the efficiency of electron flow and ADP/ATP exchange (Claypool et al., 2008b; Lapuente-Brun et al., 2013; Mileykovskaya et al., 2013; Schagger and Pfeiffer, 2000). Mammalian respiratory supercomplexes consist of one complex I, two complex IIIs, and 1-4 complex IVs (Schagger and Pfeiffer, 2000), In yeast, which lack complex I, supercomplexes consist of

two complex IIIs, and 1-2 complex IVs (Cruciat et al., 2000; Schagger and Pfeiffer, 2000).

Mitochondrial Dynamics

Mitochondria are not static organelles; instead they form an elongated network that continually fuses, divides, and moves throughout the cell. Mitochondrial fusion allows mixing of proteins and mtDNA between individual mitochondrion, minimizing the effect of ROS-mediated mutations and allowing gene complementation (reviewed in Chan, 2006; Westermann, 2010). Mitochondrial fission, on the other hand, is important for segregation of dysfunctional mitochondria which will be eliminated by mitophagy (Twig et al., 2008), and plays a role in apoptosis (reviewed in Suen et al., 2008). Disruptions in the balance of these two opposing processes lead to mitochondrial dysfunction.

Fusion of the OM requires MFN1 and MFN2 in mammals, or Fzo1p in yeast. These proteins are GTPases which form oligomeric complexes that tether adjacent membranes and mediate fusion (Griffin and Chan, 2006; Ishihara et al., 2004; Koshiba et al., 2004; Meeusen et al., 2004).

Fusion of the IM is mediated by Mgm1p in yeast, and by its mammalian ortholog, OPA1 (Delettre et al., 2000). Mgm1p is a dynamin-related GTPase that self assembles and tethers adjacent inner membranes, bringing them into close physical proximity for fusion (Meeusen et al., 2006; Smirnova et al., 1999; van der Bliek, 1999). Multiple OPA1 isoforms are generated as a result of alternative splicing and limited proteolysis by the *m*-AAA and *i*-AAA proteases (Delettre et al., 2001; Duvezin-Caubet et al., 2006; Duvezin-Caubet et al., 2007; Griparic et al., 2007; Song et al., 2007); likewise Mgm1p is

processed into long (l-Mgm1p) and short (s-Mgm1p) isoforms, which are present in roughly equal amounts. The short isoform is generated through cleavage by the rhomboid protease, Pcp1p (Herlan et al., 2003; McQuibban et al., 2003). The proper balance of the various isoforms is critical for IM fusion. (DeVay et al., 2009; Duvezin-Caubet et al., 2006; Ehses et al., 2009; Head et al., 2009; Herlan et al., 2003; Zick et al., 2009).

Mitochondrial fission is mediated by another dynamin-related protein, Dnm1p in yeast (Otsuga et al., 1998), and its ortholog DRP1 in humans (Smirnova et al., 1998). Dnm1p is a GTPase that localizes to sites of mitochondrial fission (Bleazard et al., 1999), oligomerizes (Fukushima et al., 2001) and constricts membranes in a similar manner to dynamin (Mears et al., 2011). Interestingly, one difference between dynamin spirals and Dnm1p spirals is their size. The oligomeric Dnm1p spirals are larger than those formed by dynamin to accommodate the larger and more variable size of mitochondria compared to endocytic vesicles (Ingerman et al., 2005; Mears et al., 2011). In the absence of DRP1/Dnm1p, mitochondria form elongated, highly branched networks due to continuing and unopposed mitochondrial fusion (Bleazard et al., 1999; Otsuga et al., 1998).

MITOCHONDRIAL DISEASE

Mitochondrial diseases affect at least 1 in 5000 (Schaefer et al., 2004), and as a result of their participation in numerous cellular processes, present with a range of clinical phenotypes. Depending on which genome is affected, mitochondrial disease can follow maternal (mtDNA) or mendelian (nuclear DNA) inheritance (Haas et al., 2008; Thorburn, 2004; Thorburn et al., 2004; Wallace, 1999). Because mtDNA only contains a

few genes, the majority of pediatric cases of mitochondrial disease involve defects originating in the nuclear genome (Thorburn, 2004). This allows these diseases to be studied with all of the classic advantages of the yeast system such as the ability to combine relatively easy genetic manipulations with robust biochemical approaches that collectively facilitate detailed mechanistic studies. In addition, yeast provides the added bonus of being amenable to suppressor screens and both large scale genetic and pharmacologic screens (for examples, see Glerum et al., 1996b; Hasson et al., 2010; Rinaldi et al., 1997; Schuldiner et al., 2005).

Using Yeast to Study Mitochondrial Biology and Human Disease

Yeast also provide advantages specific to studying mitochondrial diseases, perhaps the most important of which is the ability to survive on fermentable carbon sources in the absence of functional OXPHOS. Therefore, pathogenic mutations that lead to dysfunction of mitochondrial ATP production can be maintained in yeast, so long as a fermentable carbon source is available. Furthermore, the growth phenotype provides a simple method of assessing mitochondrial function; when grown in media containing only non-fermentable carbon sources such as glycerol, ethanol, or lactate, strains exhibiting OXPHOS dysfunction are unable to grow.

A large number of diseases are associated with mtDNA defects, which are caused by point mutations, rearrangements, and/or deletions (MITOMAP.org, 2011; Tuppen et al., 2010). The utility of using yeast to model these diseases is exemplified by the unique ability to directly transform yeast mtDNA (Bonnefoy and Fox, 2007; Ding et al., 2009; Fox et al., 1988). In yeast, biolistic transformation of a ρ^0 strain (a strain completely lacking mtDNA) is able to generate the desired mutant strain relatively easily. This

allows a defined mutation that may be identified in unrelated human patients to be studied in the context of one host nuclear genetic background. Of course, this capability additionally allows distinct mtDNA mutations in the same gene or different genes to be studied, compared and contrasted in the same genetic background.

In higher organisms, a limited number of mtDNA mutants have been described, most of which have been generated through indirect manipulation of mtDNA (Tyynismaa and Suomalainen, 2009; Wallace and Fan, 2009; Xu et al., 2008), although efficient complementation of a mtDNA deletion by targeting RNA to mitochondria has recently been described in mammalian tissue culture (Mahato et al., 2011).

The difficulty in generating mtDNA mutations in mammalian cells is compounded by their ability to harbor heteroplasmic mtDNA genomes. A single cell contains hundreds to thousands of individual mtDNA genomes. Normally all mtDNA copies are the same (termed "homoplasmy"), but when detrimental mutations are present, both wild type and mutant mtDNA genomes are present within the cell (termed "heteroplasmy") (DiMauro and Moraes, 1993; Wong, 2007). Unlike mammalian cells, yeast become homoplasmic within a few generations (Birky, 2001; Shibata and Ling, 2007). In mammals, a mutation in mtDNA may be present in a few copies, but not result in a clinical phenotype because the remaining wild type mtDNA is able to complement the defect. It is not until the mutant mtDNA reaches a minimum critical number that dysfunction is evident. This phenomenon, known as the threshold effect, is often ascribed to the progressive and varied onset of mitochondrial diseases and pleiotropic phenotypes (Birky, 2001; Wong, 2007). While this aspect cannot be modeled well in

yeast, the absence of additional mutants or a subpopulation of wild type genes make yeast useful in studying pathogenic mtDNA mutants in isolation.

Thus, the yeast model is not without its limitations. In addition to the inability to study the threshold effect, diseases affecting complex I represent a significant portion of respiratory chain defects and cannot be modeled in yeast since it lacks this complex. Finally, models in multicellular organisms are required to understand the tissue specific phenotypes exhibited by many mitochondrial diseases.

Despite the limitations, the conservation of mitochondrial functions between yeast and humans make yeast an invaluable model for elucidating basic mitochondrial processes, as well as providing a better understanding of mitochondrial disease (Figure 1.1). The ability to efficiently generate mutations, even within mtDNA, has provided a greater understanding of the molecular mechanisms underlying several diseases (reviewed in Baile and Claypool, 2013), which will ultimately enhance the ability to design specific therapeutic treatments. The yeast model has also proven to unambiguously distinguish between pathogenic mutations and silent polymorphisms, which is critical for appropriate genetic counseling. Finally, basic research using yeast and focused on questions of fundamental importance concerning mitochondrial biogenesis and physiology provide logical candidates for assorted mitochondrial diseases which has already, and will continue, to streamline the important task of identifying the pathogenic lesions that cause mitochondrial disease. As such, the contribution of the yeast model to the field of mitochondrial medicine is immeasurable.

Common Laboratory Yeast Strains Often Carry Mitochondrial Defects

A number of genetically distinct laboratory yeast strains have been used to study mitochondrial functions, including S288c, W303, and D273. Many of these strains carry mutations affecting mitochondrial functions. A more comprehensive list of yeast strains used in mitochondrial studies can be found in (Young and Court, 2008).

Strain S288c carries a mutation in *MIP1*, which encodes the mitochondrial DNA polymerase, consisting of an Ala to Thr substitution at the highly conserved residue 661 (termed the *MIP1*[S] allele). This alteration increases the mutation rate during mtDNA replication, especially at elevated temperature (Baruffini et al., 2007; Young and Court, 2008). S288c also contains a Ty1 element inserted into the 3' region of *HAP1* (Gaisne et al., 1999). Hap1p is a transcription factor that controls the expression of multiple oxygen-inducible genes, including some that are incorporated into respiratory complexes (Zitomer and Lowry, 1992). The Ty1 insertion greatly reduces the ability of Hap1p to induce genes under the control of its upstream activation sequence (UAS), most notably *CYC1*, the gene encoding the mobile electron carrier, cytochrome *c* (Gaisne et al., 1999). Additionally, when the derivative of S288c, BY4741/2, was analyzed, it contained mutations in *SAL1* and *CAT5* (Dimitrov et al., 2009). *SAL1* encodes the mitochondrial Mg-ATP/P_i exchanger, and *CAT5* encodes a protein required for ubiquinone (CoQ) biosynthesis. Thus, the S288c strain and its derivatives contain multiple mutations that result in less than optimal OXPHOS function. Despite this, S288c does have advantages; in addition to numerous auxotrophic markers, its genome has been completely sequenced, and the yeast knock-out collection was generated using the S288c derivative strains BY4741/2 (Giaever et al., 2002).

W303 and its descendants do not contain mutations in *HAP1* (Gaisne et al., 1999), *SAL1,* or *CAT5*, leading to higher respiration rates than S288c. However, these strains do contain the *MIP1*[S] allele, increasing mtDNA mutability (Baruffini et al., 2007). Additionally, the presence of the *ade2* allele in combination with *MIP1*[S] can further increase the rate of mtDNA point mutations (Young and Court, 2008).

The D273 strain, commonly used due to its high respiratory rate, contains the wild type *MIP1* allele (Baruffini et al., 2007), although auxotrophic markers are limited, and the lack of the 2-micron episome prevents multi-copy plasmid transformation.

Therefore, the genetic background is an important consideration when using yeast to study mitochondrial processes. Wild type strains with decreased mitochondrial function may make the assessment of mutants with partial function more difficult. On the other hand, these strains may exacerbate certain phenotypes, making it easier to screen for suppressors or genetic interactions.

THE SIGNATURE LIPID OF MITOCHONDRIA: CARDIOLIPIN

Mitochondria contain the unique phospholipid cardiolipin (CL), which is required for efficient activity of a number of mitochondrial processes (Claypool and Koehler, 2012). CL is unique for a number of reasons: 1) unlike most other phospholipids which are synthesized in one or a few cellular locations, then disseminated throughout a cell's membranes, CL by and large remains in the mitochondrion, its site of synthesis; 2) CL is essentially a lipid dimer; it consists of two phosphate headgroups attached by a glycerol moiety and four acyl chains (Pangborn, 1947); and 3) after its synthesis, CL undergoes acyl chain remodeling, where acyl chains are removed by a lipase forming monolyso-CL (MLCL) and replaced by a transacylase or acyltransferase, resulting in the establishment

of only a few molecular forms of CL in a cell or tissue. Curiously, the final molecular form of CL varies between organisms and even between cell types within the same organism (Schlame et al., 2005).

Cardiolipin and Mitochondrial Function

CL serves the cell in multiple capacities. It associates with all the major protein complexes of the mitochondrial respiratory chain, including the electron transport chain complexes and the ATP/ADP carrier (Acehan et al., 2011; Claypool et al., 2008b; Eble et al., 1990; Fry and Green, 1981; Jiang et al., 2000; Schagger et al., 1990; Schwall et al., 2012; Yu and Yu, 1980). CL optimizes both the individual catalytic activities of these complexes (Hoffmann et al., 1994; Schwall et al., 2012; Wenz et al., 2009) and facilitates the assembly of respiratory supercomplexes (Acehan et al., 2011; Bazan et al., 2013; Brandner et al., 2005; Claypool et al., 2008b; Jiang et al., 2000; Schagger and Pfeiffer, 2000; Zhang et al., 2002; Zhang et al., 2005). CL is also involved in the import of proteins translated by cytosolic ribosomes into mitochondria by maintaining a higher membrane potential required for protein import (Jiang et al., 2000) and stabilizing the import machinery (Gebert et al., 2009; Marom et al., 2009). Mitochondrial fission and fusion are affected by the absence of CL; CL is involved in the binding of Drp1p/Dmn1p to the OM for mitochondrial fission (Montessuit et al., 2010), and is required for the proper processing of the IM fusion protein OPA1/Mgm1p (Ban et al., 2010; DeVay et al., 2009). The reduced abundance or complete absence of CL results in mitochondrial cristae morphology defects (Acehan et al., 2009; Acehan et al., 2007; Claypool et al., 2006; Mileykovskaya and Dowhan, 2009). CL is implicated in apoptosis, where it can be oxidized by cytochrome *c*, mediating the release of cytochrome *c* from the IM (Kagan et

al., 2005). Its appearance on the outer leaflet of the OM serves as a docking site for caspase-8, tBID, and BAX (Garcia Fernandez et al., 2002; Gonzalvez et al., 2008; Kuwana et al., 2002; Lutter et al., 2000). More recently, CL transport to the OM has been linked to mitophagy (Chu et al., 2013), a specialized form of autophagy that selectively removes mitochondria (Youle and Narendra).

Cardiolipin and Disease

Reflecting the importance of CL in promoting and maintaining normal mitochondrial function, alterations in CL metabolism have been associated with a number of different human pathologies (reviewed in Chicco and Sparagna, 2007; Houtkooper and Vaz, 2008; Paradies et al., 2009).

Changes in phospholipids, including CL, have been observed in patients with heart disease, as well as various models of heart failure. Alterations in the molecular species of CL occurs in models of, and patients with, cardiac hypertrophy (O'Rourke and Reibel, 1992; Reibel et al., 1986), ischemic or dilated cardiomyopathy (Heerdt et al., 2002; Le et al., 2014), spontaneously hypertensive heart failure (Saini-Chohan et al., 2009; Sparagna et al., 2007; Sparagna et al., 2005; Zachman et al., 2010), and diabetic cardiomyopathy (Han et al., 2005; Han et al., 2007). The mechanisms through which CL acyl chains are altered during heart failure are presently unclear, but may be due to altered expression or activity of CL remodeling enzymes (He and Han, 2014; Le et al., 2014; Saini-Chohan et al., 2009).

During ischemia and reperfusion, CL content decreases (Lesnefsky et al., 2001; Nakahara et al., 1992; Soussi et al., 1990), and often occurs with a concurrent increase in peroxidized CL (Paradies et al., 2004; Paradies et al., 1999; Petrosillo et al., 2005;

Petrosillo et al., 2003). Thus, lower CL amounts following ischemia and reperfusion are hypothesized to be due to the oxidation and subsequent degradation of CL (McLean et al., 1993), but decreased CL synthesis may also play a role (Cheng and Hatch, 1995).

Loss of CL, increased CL peroxidation, and altered CL acyl chain composition is also associated with aging (Ames et al., 1995; Lee et al., 2006; Lewin and Timiras, 1984; Ruggiero et al., 1992; Shigenaga et al., 1994) and may contribute to aging related diseases, such as Parkinson's disease (Ellis et al., 2005).

Finally, CL remodeling is deficient in Barth syndrome. Barth syndrome patients present with dilated cardiomyopathy, neutropenia, 3-methylglutaconic aciduria, and abnormal mitochondrial function (Barth et al., 1983; Barth et al., 1996; Barth et al., 1999; Schlame and Ren, 2006). Barth syndrome is caused by mutations in the gene *TAFAZZIN* (*TAZ1*) (Bione et al., 1996), a transacylase that transfers an acyl chain from a phospholipid to a MLCL, regenerating CL (Gu et al., 2004; Xu et al., 2003; Xu et al., 2006b). In both patients with, and models of, Barth syndrome, total CL levels are decreased, MLCL accumulates, and the remaining CL contains an altered acyl chain composition (Gu et al., 2004; Schlame et al., 2003; Soustek et al., 2010; Valianpour et al., 2005; Whited et al., 2013; Xu et al., 2006a)

THE CELL BIOLOGY OF CARDIOLIPIN

Much of the knowledge of CL biosynthesis and remodeling (figure 1.2) comes from studies in yeast. In addition to the "usual" advantages of using yeast as a model system (Baile and Claypool, 2013; Botstein and Fink, 2011), yeast are viable in the absence of CL and CL precursor phospholipids (Chang et al., 1998a; Chang et al., 1998b; Jiang et al., 1997; Osman et al., 2010; Tuller et al., 1998) whereas in higher eukaryotes

CL is required for life (Zhang et al., 2011). Although CL biosynthesis and remodeling are highly conserved between yeast and higher eukaryotes, there are still a few differences (figure 1.3). There are no orthologs of Gep4p, the phosphatidylglycerolphosphate (PGP) phosphatase, or Cld1p, a CL lipase, in higher eukaryotes (Beranek et al., 2009; Osman et al., 2010). However, the phylogenetically unrelated PTPMT1 performs the same function as Gep4p (Zhang et al., 2011); and a calcium-independent phospholipase A_2 has been implicated as a CL lipase (Malhotra et al., 2009; Mancuso et al., 2007; Schlame et al., 2012b), although its exact role in CL remodeling remains nebulous (Kiebish et al., 2013). Additionally, only the Taz1p-mediated CL remodeling pathway exists in yeast, while additional remodeling enzymes have been identified in mammals: MLCLAT-1/TFPα has been shown to bind MLCL and affect the acyl chain composition of CL, preferentially adding 18:2 fatty acids to MLCL (Taylor and Hatch, 2003; Taylor and Hatch, 2009; Taylor et al.2012); and ALCAT1, an ER resident protein, has been shown to generate CL with very long chain fatty acids, and its overexpression results in mitochondrial dysfunction (Cao et al., 2004; Cao et al., 2009; Li et al., 2010). However, the relative contribution of these additional CL remodeling enzymes has recently been challenged (Schlame et al., 2012b). Thus, while yeast have been useful in dissecting this process, the complexity of and multitude of players in mammalian CL remodeling suggest that there is still much to discover.

With the recent characterizations of Cld1p, Gep4p and Tam41p (Beranek et al., 2009; Osman et al., 2010; Tamura et al., 2013), it is likely that all of the proteins catalyzing CL synthetic or remodeling reactions have been identified in yeast; however,

many questions regarding the regulation of this process, as well as the topology and trafficking of CL and its precursors, remain (Figure 1.2).

Cardiolipin Precursors

CL biosynthesis requires CDP-diacylglycerol (CDP-DAG), which is formed from phosphatidic acid (PA) and CTP by a CDP-DAG synthase (Shen et al., 1996). Yeast contain two CDP-DAG synthases: Cds1p in the ER (Kuchler et al., 1986), and the recently characterized Tam41p in the mitochondrial IM (Tamura et al., 2013).

Although CDP-DAG (containing an NBD moiety) is able to be translocated from the ER to the IM *in vitro*, this process is inefficient (Tamura et al., 2013). The very low abundance of CL in Δ*tam41* yeast (Kutik et al., 2008; Tamura et al., 2012) suggests that if Cds1p-derived CDP-DAG contributes to CL biosynthesis, its role is very minor. Tam41p is peripherally associated with the matrix side of the IM (Table 1.1) (Gallas et al., 2006; Tamura et al., 2013). Thus, Tam41p activity requires that its substrate, PA, be transported from the ER to the matrix-facing leaflet of the IM. Phospholipid transport between the ER and mitochondrial OM was suggested to be mediated by the ER-mitochondria encounter structure (ERMES) complex which physically tethers the two organelles (Kornmann et al., 2009). Indeed, loss of any ERMES complex subunit (Mdm10p, Mdm34, Mdm12p, or Mmm1p) alters the mitochondrial phospholipid profile, including reducing CL (Kornmann et al., 2009; Stroud et al., 2011; Tamura et al., 2012). However, a direct role for ERMES in phospholipid transport has recently been challenged (Nguyen et al., 2012; Voss et al., 2012). Furthermore, defects caused by the loss of a functional ERMES complex can be rescued by expressing an artificial ER-mitochondria tether, suggesting that the ERMES complex facilitates phospholipid

transport by forming close contact sites between the two membranes, rather than directly transporting phospholipids (Kornmann et al., 2009; Nguyen et al., 2012; Voss et al., 2012). Notably, these studies focused on the transport of phosphatidylserine from the ER to mitochondria (and phosphatidylethanolamine to the ER after is decarboxylation in mitochondria). Thus, the mechanisms of PA and CDP-DAG transport from the ER to mitochondria, and the players involved, including a direct assessment of the role of the ERMES complex, remain to be discovered.

To reach the IM, CL precursor phospholipids must traverse the OM, but little is known about this process. Phospholipid exchange between leaflets of purified OM vesicles is rapid, suggesting that proteins mediate this process. However, treatment with proteases or with sulfhydryl reactive compounds does not inhibit transbilayer movement across the OM (Janssen et al., 1999).

PA is transported from the OM to the IM by the IMS resident protein, Ups1p (Connerth et al., 2012). Mdm35p binds Ups1p, facilitating its import into the IMS and preventing its proteolytic degradation (Potting et al., 2010; Tamura et al., 2010). Although Ups1p/Mdm35p dimers can bind negatively charged phospholipids, only PA is transported *in vitro*, demonstrating the specificity of its transport activity.

Once delivered to the IM, PA must traverse to the matrix side of the IM. This could be accomplished by an unidentified protein or alternatively, PA may redistribute to both leaflets of the IM based on the transmembrane pH gradient (Gallet et al., 1999; Hope et al., 1989).

Synthesizing Cardiolipin

The first committed step of CL biosynthesis is the formation of PGP from CDP-DAG and glycerol-3-phosphate by Pgs1p (Chang et al., 1998a). While the topology of Pgs1p has never been formally investigated (Table 1.1), the presence of an N-terminal presequence, which is able to import the *lacZ* gene product to the matrix (Dzugasova et al., 1998), suggests that Pgs1p is localized on the matrix side of the IM. PGP is then dephosphorylated to phosphatidylglycerol (PG) by Gep4p, a protein peripherally attached to the matrix side of the IM (Osman et al., 2010). In the final step of CL biosynthesis, PG and another CDP-DAG are condensed to form CL by Crd1p (Chang et al., 1998b; Jiang et al., 1997). Characterization of the rat Crd1p homolog from liver indicates that it is an integral membrane protein and that its active site faces the matrix (Gallet et al., 1997; Schlame and Haldar, 1993).

Remodeling Cardiolipin

CL remodeling is initiated by the lipase Cld1p in yeast (Beranek et al., 2009), which removes an acyl chain from CL, generating MLCL. Taz1p performs an acyl-CoA independent transacylation reaction, transferring an acyl chain from a phospholipid to a MLCL, regenerating CL (Gu et al., 2004; Xu et al., 2003; Xu et al., 2006b).

Surprisingly, the localization of Taz1p is not the same as enzymes upstream in the pathway. In yeast, Taz1p was originally localized to the mitochondrial OM (Brandner et al., 2005), but was later shown to be present on both the IM and OM, on leaflets facing the IMS (Claypool et al., 2006; Gebert et al., 2009). Taz1p is an interfacial membrane protein; it contains residues that are embedded in, but not through, the membrane (Claypool 2006).

When this thesis began, the localization of Cld1p within mitochondria was unknown. Regardless of the localization of Cld1p however, after its synthesis on the matrix side of the IM, CL must traverse the IM or be transported to the OM to become accessible to Taz1p and complete remodeling. To gain a better understanding of this translocation process during CL remodeling, Cld1p was characterized, the results of which are presented in Chapter 2.

Independent of the precise point during remodeling that CL is translocated, the mechanism of CL translocation remains undefined. While proteins mediating phospholipid redistribution between membrane leaflets have been identified for the plasma membrane, Golgi, and endosomes (van Meer et al., 2008), considerably less is known about this process in the mitochondrion. CL redistribution between IM leaflets has been observed (Gallet et al., 1997; Gallet et al., 1999), but the protein(s) responsible has not been identified. So far, phospholipid scramblase 3 (PLS3) is the only mitochondrial protein suggested to facilitate transbilayer lipid trafficking (Liu et al., 2003), but this has not been formally demonstrated. Importantly, the translocation of phospholipids between membrane leaflets may not be facilitated by specific proteins, but instead non-specifically by the presence of numerous transmembrane proteins, as has been suggested for bacterial membranes and the ER (Kol et al., 2004; Kol et al., 2001; van Meer et al., 2008).

Establishing the Final Distribution of Cardiolipin

CL is enriched in the IM, but is also present on the OM (Gebert et al., 2009). How it achieves its final distribution in yeast is still unclear. Intriguingly, the presence of a subpopulation of Taz1p on the OM opens up the possibility that MLCL may be the lipid

species trafficked from the IM to the OM, where it is then reacylated to form CL (Claypool et al., 2006; Gebert et al., 2009).

Phospholipid transfer between the OM and IM has been suggested to occur at contact sites between the two membranes (Blok et al., 1971; Simbeni et al., 1990). Recently, the proteins comprising this complex (termed MINOS, mitochondrial IM organizing system, MitOS, or MICOS) have been identified (Harner et al., 2011; Hoppins et al., 2011; von der Malsburg et al., 2011). Loss of this complex results in abnormal cristae morphology and loss of cristae junctions. However, the effects on phospholipid transport, the import of the CL precursor PA, or the final distribution of CL, have yet to be studied in contact site mutant yeast strains.

Currently, three proteins have been described that have the ability to traffic/redistribute CL in mammals: the mitochondrial creatine kinase (MtCK) (Epand et al., 2007), the mitochondrial nucleoside diphosphate kinase (NDPK-D) (Epand et al., 2007; Schlattner et al., 2013), and phospholipid scramblase 3 (PLS3) (Liu et al., 2003). However, MtCK has no ortholog in yeast. Yeast also do not contain a mitochondrial-specific ortholog of NDPK-D, although a small portion of the yeast nucleoside diphosphate kinase, Ynk1p, localizes to mitochondria (Amutha and Pain, 2003). However, if Ynk1p can transport CL between membranes remains to be determined. PLS3 was shown to redistribute CL between the IM and OM in mammalian mitochondria (Liu et al., 2003), but phospholipid transport between membranes is inconsistent with its role as a scramblase. Thus, it is likely that PLS3 instead coordinates with a CL transport protein, and the altered distribution of CL between the OM and IM when PLS3 is

overexpressed reflects the increased availability of CL on the IMS-facing leaflet of the IM.

Three UPS isoforms exist in yeast, although a concrete function has yet to be assigned to Ups2p or Ups3p. It is tempting to speculate that, like the PA transport function of Ups1p (Connerth et al., 2012), Ups2p and Ups3p can transport CL (or MLCL) between the IM and OM. Interestingly, total CL levels in Δ*ups2* and Δ*ups3* mutants remain largely unaffected (Osman et al., 2009; Tamura et al., 2009), but the relative distribution of CL between the IM and OM has never been analyzed.

Regulation of Cardiolipin

Consistent with its role in mitochondrial processes, CL levels change depending on the energetic requirements of a cell, and its synthesis is regulated at several different points during its biosynthesis (figure 1.4). Once PA reaches the OM, its transport can be regulated by CL levels. The PA transporter Ups1p is unable to dissociate from membranes containing physiological levels of CL. Thus, the higher amount of CL in the IM is modeled to not only confer directionality of PA transport, but also to limit the accumulation of CL (Connerth et al., 2012).

Exogenous inositol downregulates phosphatidylcholine and phosphatidylinositol biosynthesis through transcriptional repression via an inositol sensitive upstream activating sequence (UAS_{INO}) (Henry et al., 2012). Pgs1p activity is similarly reduced in the presence of inositol, but it contains a mutated, nonfunctional UAS_{INO} sequence (Bachhawat et al., 1995) and *PGS1* mRNA levels are unchanged in the presence of inositol (Zhong and Greenberg, 2003). Further, deletion of the UAS_{INO}-binding genes *INO2, INO4,* or *OPI1* does not affect Pgs1p activity (Greenberg et al., 1988), suggesting

its inositol-mediated regulation is independent of the *INO2-INO4-OPI1* circuit. Indeed, inositol increases Pgs1p phosphorylation, leading to its repressed activity, although the kinase(s) involved has yet to be identified (He and Greenberg, 2004). Independent from its inositol-mediated regulation, Pgs1p activity is increased under conditions indicative of mitochondrial biogenesis; its mRNA abundance is highest when cells enter stationary phase, and its activity is higher in the presence of non-fermentable carbon sources and when cells contain functional mtDNA (Gaynor et al., 1991; Shen and Dowhan, 1998; Zhong and Greenberg, 2003).

Crd1p activity is similarly increased during stationary growth, in the presence of mtDNA, and in the presence of non-fermentable carbon sources, leading to increased CL levels (Baile et al., 2013; Claypool et al., 2008a; Gaynor et al., 1991; Jakovcic et al., 1971; Jiang et al., 1999; Su and Dowhan, 2006). This observation is not surprising considering the importance of CL in a myriad of mitochondrial functions (Claypool and Koehler, 2012); as mitochondrial biogenesis increases, CL levels concurrently increase.

Crd1p activity can be additionally regulated by the matrix pH (Gohil et al., 2004). Treatment of yeast with the protonophore CCCP (which disrupts the pH and electrical gradients across the IM), but not the K^+ ionophore valinomycin (which disrupts the electrical gradient but does not affect matrix pH), decreases Crd1p activity. A decrease in the matrix pH is indicative of less robust electron transport chain activity, coordinating the mitochondrion's energetic requirements with CL biosynthesis. This is further exemplified by the decreases in CL levels that result from defects in respiratory complexes and/or bioenergetic function (Gohil et al., 2004; Zhao et al., 1998).

Interestingly, while steady state CL levels were reduced in Δ*taz1* yeast, synthesis of CL was actually increased in the mutant, concurrent with MLCL accumulation (Gu et al., 2004). These observations led the group to suggest that *de novo* CL biosynthesis might be regulated by downstream CL acylation/remodeling. Crd1p's activity might therefore be negatively regulated by its own product, and under conditions where CL is decreased or aberrantly acylated, the cell compensates by promoting CL biogenesis.

CL biosynthesis is thus regulated via multiple independent mechanisms: by inositol, which regulates Pgs1p; by mitochondrial biogenesis, which affects Pgs1p and Crd1p activity; by CL, which may inhibit Crd1p activity; and by the capacity for oxidative phosphorylation, which affects Crd1p.

Unlike CL biosynthesis, which has been extensively studied and for which multiple modes of regulation have been identified, virtually nothing was known about the regulation of CL remodeling, or even if CL remodeling is regulated, when this thesis began. Additionally, how the final acyl chain composition is determined is currently unknown. Acyl chain composition could be conferred by the lipase, but its acyl chain specificity has never been demonstrated (Beranek et al., 2009). Further, the transacylase tafazzin has no acyl chain specificity *in vitro* (Schlame, 2012), although tafazzin from *Drosophila* has been shown to preferentially catalyze transacylation reactions on curved membranes leading to the establishment of CL with unsaturated acyl chains, which were proposed to decrease lipid disorder in areas of high curvature (Schlame et al., 2012a). In Chapter 2, we provide two independent regulatory mechanisms of CL remodeling, and provide evidence that suggests that Cld1p contributes to CL acyl chain composition.

The Physiological Role of Cardiolipin Remodeling

Despite the well-defined role of perturbed CL remodeling in disease (Chicco and Sparagna, 2007; Claypool and Koehler, 2012; Han et al., 2007; Lesnefsky et al., 2009; Schlame and Ren, 2006; Sparagna and Lesnefsky, 2009; Vreken et al., 2000), the contribution of CL remodeling to normal mitochondrial function remains enigmatic. The tafazzin-mediated remodeling pathway is evolutionarily conserved. Tafazzin orthologs are conserved from yeast to humans; contrastingly, Cld1p is present in yeast (Beranek et al., 2009), but an ortholog has not been identified in metazoans. However, the transacylase activity of tafazzin requires a lysophospholipid (Xu et al., 2006b); thus a lipase, albeit currently unidentified, functions upstream of tafazzin in metazoans. Regardless of the identity of the initiating lipase, the conservation of this pathway suggests that it serves some important function(s).

Different organisms and even different cell types within the same organism contain different molecular species of CL (Cheng et al., 2008; Han et al., 2006; Schlame et al., 2005). This, combined with the observation that mitochondria from different tissues contain different proteomes (Mootha et al., 2003; Pagliarini et al., 2008), contributes to the prevailing hypothesis that CL remodeling functions to establish a specific molecular form of CL that is tailored to meet the individual requirements of the host cell/organism. Consistent with this, the CL that remains in Barth syndrome patients contain an altered acyl chain composition (Houtkooper et al., 2009; Schlame et al., 2005; Valianpour et al., 2005; Vreken et al., 2000), and has been suggested to contribute to mitochondrial dysfunction in those patients (Houtkooper et al., 2009; Schlame and Ren, 2006; Schlame et al., 2005; Vreken et al., 2000). However, the hypothesis that

remodeled CL is better suited to promote mitochondrial function has never been formally tested in otherwise isogeneic cells, and is the subject of Chapter 3.

TABLES

Table 1.1. Topology of CL synthesis and remodeling enzymes

Protein	Location/ membrane association	Predicted transmembrane domains[a]	Biochemical experiments	References
Tam41p	Matrix leaflet of IM/peripheral	0	Protected from protease in mitoplasts, extracted with carbonate	(Tamura et al., 2006)
Pgs1p	Matrix leaflet of IM/peripheral	0, 1, 2[b]	N-terminal presequence imports *LacZ* to mitochondrial matrix	(Dzugasova et al., 1998)
Gep4p	Matrix leaflet of IM/peripheral	0	Protected from protease in mitoplasts; extracted with carbonate	(Osman et al., 2010)
Crd1p	Active site faces the matrix of the IM/integral	2, 3, 4, 5	Protected from protease in mitoplasts; blocking divalent cation entry into the matrix inhibits CL synthesis[c]	(Schlame and Haldar, 1993)
Cld1p	See Chapter 2	0, 1, 2		
Taz1p	IMS-facing leaflet of IM and OM/non-integral	0, 1, 2	Degraded by protease in mitoplasts; partially extracted with carbonate; epitope tags throughout the polypeptide face the IMS	(Claypool et al., 2006)

[a]Transmembrane predictions were determined using the DAS-TMfilter prediction server (Cserzo et al., 2004), TMpred (Hofmann and Stoffel, 1993), HMMTOP (Tusnady and Simon, 1998), TMHMM (Krogh et al., 2001), and SPLIT (Juretic et al., 2002)

[b]Most programs predicted Pgs1p to have 0 transmembrane domains, except TMpred which predicted either 1 or 2 transmembrane segments

[c]Biochemical experiments have not been performed on yeast Crd1p. The experiments here analyzed the rat Crd1p homolog

FIGURES

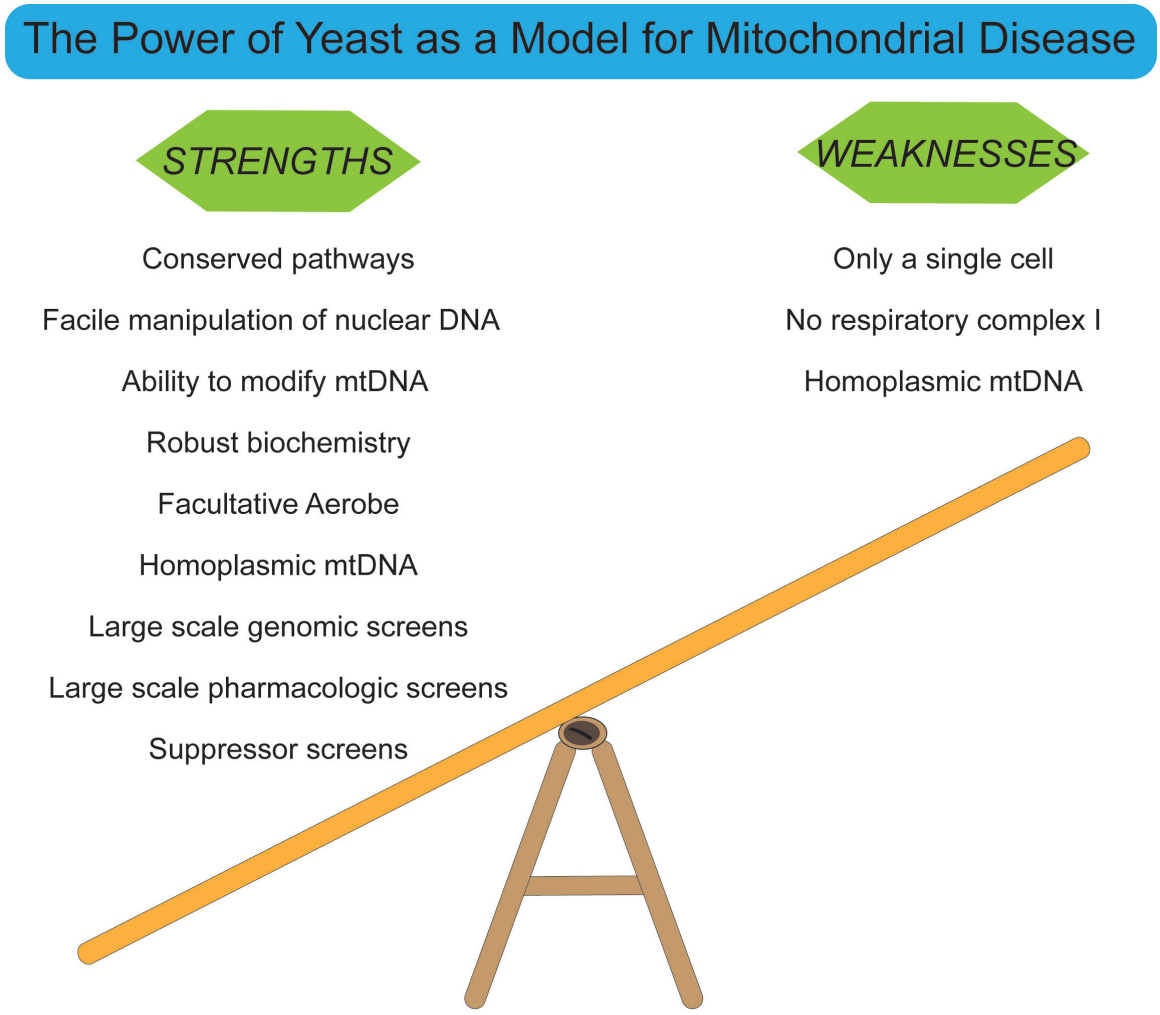

Figure 1.1. The power of yeast as a model for human mitochondrial disease. When the weaknesses of the yeast model (unicellular, absence of respiratory complex I, and homoplasmic mtDNA) are directly compared to its strengths (high degree of conservation of basic mitochondrial processes, easy genetics, robust biochemistry, ability to survive in the absence of a functional OXPHOS system, homoplasmic mtDNA, and capacity for large scale genetic, pharmacologic, and suppressor screens) with respect to modeling human mitochondrial disease, the value of yeast is clearly evident.

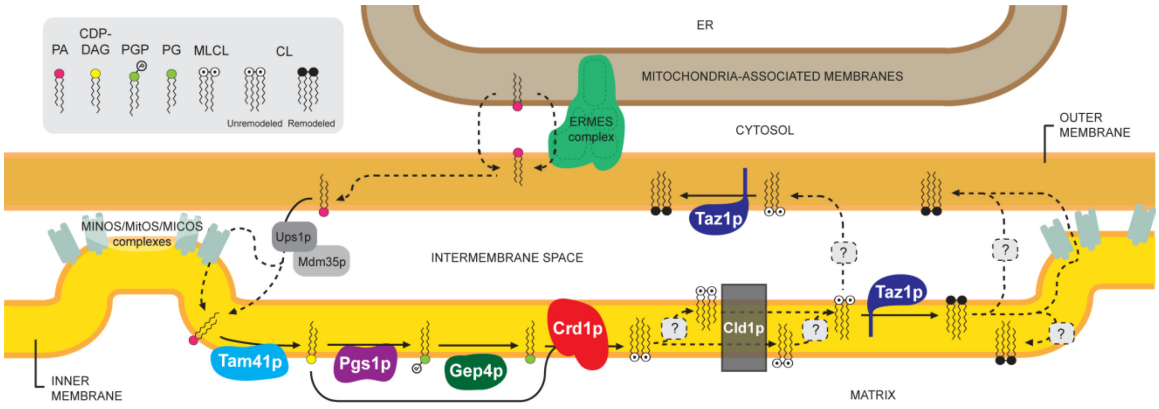

Figure 1.2. The topology of CL biosythesis and remodeling in yeast. Phosphatidic acid (PA) is synthesized in the ER and translocates to mitochondria in a process that is influenced by the ERMES (ER-mitochondria encounter structure) complex. Ups1p/Mdm35p heterodimers transport PA from the OM to the IM, potentially at contact sites (established by MINOS/MICOS/MitOS complexes). PA is converted to CDP-diacylglycerol (CDP-DAG) by Tam41p on the matrix-facing leaflet of the IM. CDP-DAG is used to generate phosphatidylglycerolphosphate (PGP) by Pgs1p. PGP is dephosphorylated to phosphatidylglycerol (PG) by Gep4p. PG and another CDP-DAG are condensed to form unremodeled CL by Crd1p. CL is deacylated by Cld1p forming MLCL. Taz1p, located on the IMS-facing leaflets of the IM and OM reacylate MLCL, which regenerates CL. Multiple rounds of deacylation/reacylation result in remodeled CL which is enriched in unsaturated acyl chains. CL achieves its final distribution on both leaflets of the IM and OM through currently ill-defined mechanisms. The depicted topology of Pgs1p has not been experimentally verified. Solid lines indicate known pathways. Dashed lines delineate potential but currently unknown phospholipid transport processes.

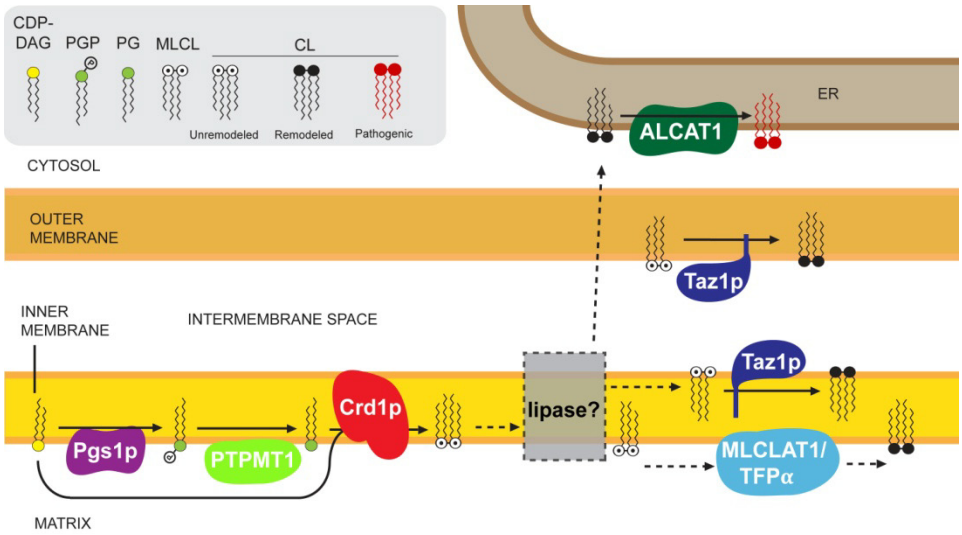

Figure 1.3. CL biosynthesis and remodeling in metazoans. Much of the CL biosynthesis and remodeling pathway is conserved between yeast and metazoans, with a few exceptions. The PG phosphatase is PTPMT1 (phylogenetically unrelated to Gep4). The CL lipase remains undiscovered in metazoans. There are two additional enzymes able to remodel CL: MLCLAT1/TFPα, which resides on the matrix side of the IM and may contribute to the final molecular form of CL; and ALCAT1, which resides in the ER and produces detrimental molecular forms of CL.

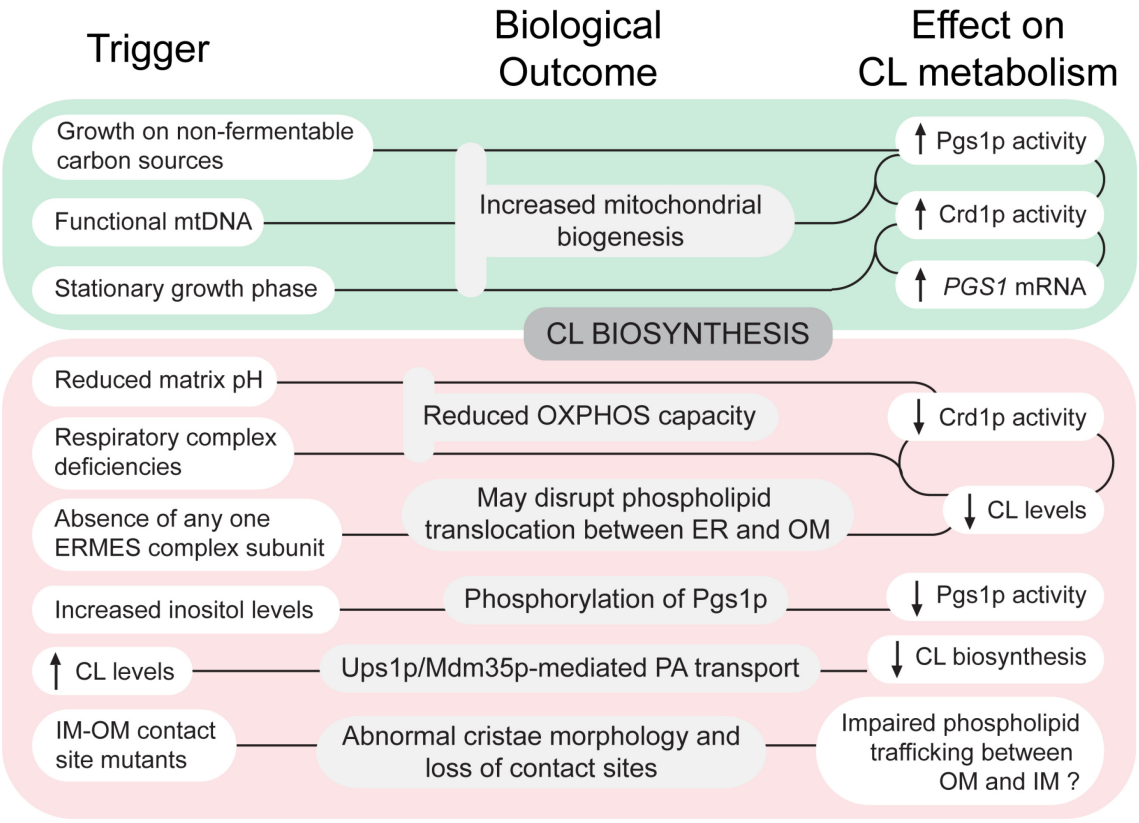

Figure 1.4. Regulatory mechanisms of CL biosynthesis. The CL biosynthetic pathway is upregulated under conditions favoring mitochondrial biogenesis. In contrast, deficiencies in ERMES (ER-OM), MINOS/MitOS/MICOS (OM-IM contact sites) complexes, and components of the electron transport chain, as well as increased levels of inositol and reduced matrix pH, can all lead to a down-regulation of CL biosynthesis. Additionally, CL levels can be modulated by Ups1p/Mdm35p-mediated PA transport. Green boxes indicate conditions that promote CL biosynthesis while red boxes indicate conditions that repress CL biosynthesis.

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Chapter 2

Deacylation on the Matrix Side of the Mitochondrial Inner Membrane Regulates Cardiolipin Remodeling

This work adapted from:

Baile, M. G., Whited, K., and Claypool, S. M. (2013) Deacylation on the matrix side of the mitochondrial inner membrane regulates cardiolipin remodeling. *Mol Biol Cell* **24**, 2008-2020

SUMMARY

The mitochondrial-specific lipid cardiolipin is required for numerous processes therein. After its synthesis on the matrix-facing leaflet of the inner membrane, cardiolipin undergoes acyl chain remodeling to achieve its final form. In yeast, this process is completed by the transacylase tafazzin, which associates with intermembrane space-facing membrane leaflets. Mutations in *TAZ1* result in the X-linked cardiomyopathy Barth syndrome. Amazingly, despite this clear pathophysiological association, the physiological importance of CL remodeling is unresolved. Here, we show that the lipase initiating cardiolipin remodeling, Cld1p, is associated with the matrix-facing leaflet of the mitochondrial inner membrane. Thus, monolysocardiolipin generated by Cld1p must be transported to intermembrane space-facing membrane leaflets to gain access to tafazzin, identifying a previously unknown step required for cardiolipin remodeling. Additionally, we show that Cld1p is the major site of regulation in cardiolipin remodeling; and that, like cardiolipin biosynthesis, cardiolipin remodeling is augmented in growth conditions requiring mitochondrially produced energy. However, unlike cardiolipin biosynthesis, dissipation of the mitochondrial membrane potential stimulates cardiolipin remodeling, identifying a novel feedback mechanism linking cardiolipin remodeling to oxidative phosphorylation capacity.

INTRODUCTION

Cardiolipin (CL) is a phospholipid unique to mitochondria that consists of two phosphatidyl head groups, bridged by a glycerol, and four total fatty acyl chains (Schlame et al., 2000). CL is important for numerous mitochondrial processes (Claypool and Koehler, 2012). It physically associates with, and enhances the function of, all of the major components of oxidative phosphorylation (OXPHOS) (Eble et al., 1990; Fry and Green, 1980; Fry and Green, 1981; Gomez and Robinson, 1999; Schwall et al., 2012; Sedlak and Robinson, 1999); promotes the stability of respiratory supercomplexes (Acehan et al., 2011; Brandner et al., 2005; Claypool et al., 2008b; Pfeiffer et al., 2003; Zhang et al., 2002; Zhang et al., 2005); is required for the optimal function of the mitochondrial fission and fusion machinery (Ban et al., 2010; DeVay et al., 2009; Montessuit et al., 2010); is involved in protein import (Gebert et al., 2009; Jiang et al., 2000; Marom et al., 2009; van der Laan et al., 2007); and is implicated in apoptosis (Gonzalvez et al., 2008; Ostrander et al., 2001). While CL is required in mammals for life (Zhang et al., 2011), it is nonessential in yeast (Jiang et al., 1997; Tuller et al., 1998). Nonetheless, yeast lacking CL display numerous defects, especially when grown in sub-optimal conditions (Jiang et al., 2000; Koshkin and Greenberg, 2000; Zhong et al., 2004).

Eukaryotic CL biogenesis is evolutionarily conserved and only occurs within mitochondrial membranes (Schlame and Haldar, 1993). Despite the presence of CL on both leaflets of the inner membrane (IM) as well as on the outer membrane (OM) (Connerth et al., 2013; Gebert et al., 2009; Petit et al., 1994), newly synthesized CL is produced within the matrix-facing leaflet of the IM (Dzugasova et al., 1998; Osman et al., 2010; Schlame and Haldar, 1993). Thus, the final distribution of CL within

mitochondrial membranes must involve trafficking steps; however the players and mechanisms responsible for these processes are presently unknown.

Furthermore, newly synthesized, immature CL, characterized by saturated acyl chains of variable length and asymmetry about the central carbon of the bridging glycerol (Schlame and Ren, 2006; Schlame et al., 2005), undergoes substantial acyl chain remodeling. CL remodeling is initiated by a phospholipase that removes an acyl chain forming monolysocardiolipin (MLCL); MLCL is then reacylated by an acyltransferase or a transacylase to form mature CL, characterized by unsaturated acyl chains and a high degree of molecular symmetry (Claypool and Koehler, 2012; Schlame et al., 2005).

At least three distinct CL remodeling pathways may exist in mammals. Interestingly, all three implicated CL remodeling enzymes are located in separate compartments. MLCL acyltransferase 1 resides on the inner leaflet of the IM (Taylor and Hatch, 2009), and acyl-CoA:lysocardiolipin acyltransferase-1 is located in the mitochondria-associated membrane compartment of the endoplasmic reticulum (ER) (Li et al., 2010). Tafazzin (Taz1p in yeast), the only CL remodeling enzyme identified in yeast (Claypool and Koehler, 2012), is a MLCL transacylase which removes an acyl chain from another phospholipid (preferentially phosphatidylcholine or phosphatidylethanolamine) and adds it to MLCL, thus regenerating CL (Xu et al., 2003; Xu et al., 2006). It is an interfacial membrane protein that resides in the intermembrane space (IMS)-facing leaflet of both the IM and OM (Claypool et al., 2006). Therefore, CL remodeling must involve not only the transbilayer movement of CL and/or its derivatives, but additionally trafficking between the IM and OM and the mitochondrion and the ER. Nothing is currently known about any of these processes.

Mutations in *TAZ1* cause the X-linked disease, Barth syndrome, which is clinically characterized by cardiomyopathy, skeletal myopathy, growth retardation and cyclic neutropenia (Barth et al., 1983; Bione et al., 1996; Schlame and Ren, 2006). Mitochondria from Barth syndrome patients exhibit abnormal ultrastructure accompanied by variable respiratory chain defects (Acehan et al., 2009; Acehan et al., 2007; Barth et al., 1983). In Barth syndrome, CL levels are decreased with a concurrent increase in MLCL, and the acyl chain composition of CL is abnormal (Schlame et al., 2003; Valianpour et al., 2005). Combined, these observations suggest that CL remodeling is required for optimal mitochondrial function (Cheng et al., 2008; Claypool and Koehler, 2012; Schlame et al., 2005).

One approach towards delineating the trafficking steps required for the biosynthesis of phospholipids is to define the subcellular localization and membrane topology of every participating enzyme. As one example, the localization of Cho1p/Pss1p to the ER and the mitochondrial Psd1p to the mitochondrial IM demonstrated that phosphatidylserine produced in the ER must traffic from the ER to the OM and finally to the IM to be decarboxylated to phosphatidylethanolamine (Horvath et al., 2012; Kuge and Nishijima, 2003; Tamura et al., 2012; Voelker, 2005). Thus, basic cell biological information outlined the steps required for the mitochondrial production of phosphatidylethanolamine. Importantly, such information also provided insight into the regulation of mitochondrial phosphatidylethanolamine production (Kuge and Nishijima, 2003).

The pathophysiological importance of the tafazzin-mediated CL remodeling pathway is firmly established (Bione et al., 1996; Schlame and Ren, 2006). In contrast,

the contribution of CL remodeling to physiology is perhaps surprisingly unresolved. In large part this reflects the absence of any information concerning if and how the tafazzin-mediated CL remodeling pathway is regulated. A major obstacle preventing a detailed investigation into the regulation of the tafazzin-mediated CL remodeling pathway is the absence of any basic cell biological information about the lipase that functions upstream of tafazzin and initiates the CL remodeling process. As such, here we sought to characterize CL-specific deacylase 1 (Cld1p), the phospholipase that initiates CL remodeling in yeast (Beranek et al., 2009), in an effort to define the trafficking steps required for tafazzin-mediated CL remodeling and gain insight into the regulation of this process. We show that endogenous Cld1p is embedded in the matrix-facing leaflet of the IM, the opposite side of the IM as Taz1p. The non-integral nature of Cld1p's membrane association indicates that Cld1p-mediated deacylation of CL to MLCL is not directly coupled to the flipping of MLCL to the IMS-facing leaflet of the IM. This in turn suggests the existence of an as yet unidentified protein(s) capable of redistributing MLCL, and possibly CL, between leaflets of the IM. Finally, we investigated the regulation of CL remodeling and demonstrate that Cld1p is the key step that controls the tafazzin-mediated CL remodeling pathway. Interestingly, disruption of the electrochemical gradient across the IM increases Cld1p function suggesting a novel mechanism by which mitochondria are able to respond to insufficient energy production by generating a form of CL that promotes and/or preserves OXPHOS capacity.

RESULTS

Cld1p resides in the mitochondrial inner membrane

Analysis of mitochondrial phospholipids indicates that Cld1p functions upstream of Taz1p in CL remodeling, however the growth phenotypes of Δ*taz1*, Δ*cld1*, and Δ*taz1*Δ*cld1* on respiratory media suggest that Cld1p may also participate in a separate pathway (Beranek et al., 2009). Thus the subcellular localization of endogenous Cld1p was analyzed. Consistent with the localization of Cld1p-green fluorescent protein (Beranek et al., 2009), endogenous Cld1p co-fractionated entirely with the mitochondrial marker, Qcr6p (Figure 2.1A) and is therefore exclusively localized to mitochondria.

Because of the topological complexity of CL remodeling (Claypool and Koehler, 2012), the submitochondrial localization of Cld1p was examined. First, we took advantage of the fact that varying concentrations of digitonin are able to selectively solubilize different mitochondrial compartments (Glick et al., 1992; Tamura et al., 2012). At very low concentrations the OM is permeabilized, releasing soluble IMS proteins (Figure 2.1B and C). As the digitonin concentration increases, the IM becomes permeabilized releasing soluble matrix proteins, followed by the solubilization and release of membrane-associated OM proteins, and finally IM proteins. The fractionation profile of Cld1p is similar to that of the IM marker proteins, indicating that Cld1p localizes to the IM.

To determine the side of the IM on which Cld1p is located, the protease accessibility of Cld1p in intact mitochondria, mitoplasts generated by osmotically rupturing the OM, and deoxycholate-solubilized mitochondrial extracts was examined. Cld1p was protected from the protease unless the IM was solubilized, similar to the

matrix protein Kgd1p; and unlike Tom70p, which faces the cytosol, or Taz1p and Dld1p, which face the IMS (Figure 2.1D). This supports the IM localization of Cld1p and additionally indicates that it either faces the matrix or, if it is a membrane spanning protein, contains an IMS-facing protease resistant domain.

Cld1p is associated with the matrix-facing leaflet of the IM

Protein topology prediction programs vary in their assessment of Cld1p, predicting 0, 1, or 2 transmembrane domains (Table 2.1). Therefore, the submitochondrial localization of each terminus of Cld1p was assessed by protease protection. Cld1p with a CNAP tag (His_{10}+Protein C (PC) epitope tags) (Claypool et al., 2008b) either on the predicted mature N-terminus (after amino acid 41) or the C-terminus were transformed into Δ*cld1*Δ*taz1* yeast to test their physiological relevance. In Δ*taz1* yeast, MLCL accumulates at the expense of CL, whereas in Δ*cld1* and Δ*cld1*Δ*taz1* yeast, CL levels are normal (Beranek et al., 2009). Thus, if Δ*cld1*Δ*taz1* yeast are rescued with a functional Cld1p, MLCL will accumulate. Both tagged forms of Cld1p were expressed (Figure 2.2A), and resulted in the accumulation of MLCL (Figure 2.2B), indicating that the addition of the CNAP tag to either terminus did not preclude function.

The CNAP tag was only degraded by the protease after the addition of detergent, regardless of its location on the mature N- or C-terminus (Figure 2.2C and D). Thus, both termini face the matrix, consistent with Cld1p containing 0 or an even number of transmembrane domains.

To experimentally determine if Cld1p is a peripheral or integral membrane protein, mitochondria were incubated in 0.1M carbonate at increasing pH. After ultracentrifugation, the integral membrane protein Pic1p remained associated with the

membrane pellet, while the peripheral membrane protein Cyc1p was released into the supernatant at every tested pH (Figure 2.3A and B). Cld1p was partially released from the membrane at pH 10.5, and was further extracted as the pH increased. This extraction profile, intermediate to either integral membrane proteins or peripheral proteins, suggests that Cld1p is an interfacial membrane protein, containing segments that extend into, but not completely through, the membrane.

The membrane association of Cld1p was further analyzed by sonication (Figure 2.3C). Mitoplasts were incubated with various concentrations of KCl. After removal of the IMS proteins, mitoplasts were resuspended in buffer containing the same concentrations of KCl and subjected to sonication. Membrane bound proteins were then separated from released proteins by ultracentrifugation. As expected, the soluble IMS protein Cyb2p was released from mitoplasts, regardless of the KCl concentration (Figure 2.3D). Cyc1p on the other hand, which is peripherally attached to the IMS-facing leaflet of the IM, remained bound to mitoplasts when no KCl was added, but was released as the KCl concentration increased. The integral membrane proteins Rip1p and Aac2p remained associated with the membrane pellet after sonication, whereas the soluble matrix protein Aco1p was released after sonication. Upon sonication, in the absence of KCl, Cld1p remained completely membrane bound but was released in a salt-titratable manner. Therefore, electrostatic interactions at least partially define Cld1p membrane association. However, that a small amount of Cld1p remained membrane bound even at very high salt concentrations indicates that electrostatic interactions are not the only determinant of its membrane association. Importantly, Cld1p was never detected in the IMS fractions regardless of the inclusion of KCl. Thus, the ability of salt to release

Cld1p from the IM only after sonication confirms that Cld1p associates with the matrix-facing leaflet of the IM. Together with the localization of the N- and C-termini of Cld1p, these results indicate that Cld1p associates with membranes but lacks canonical membrane spanning segments.

The membrane association of Cld1p is enhanced by its substrate

The role of CL in the membrane association of Cld1p was next tested in mitochondria lacking CL synthase (Δ*crd1*), and thus CL. After sonication, Cld1p remained mostly membrane bound in the absence of KCl, even in Δ*crd1* mitochondria (Figure 2.3E and F). However, at both 100 and 250 mM KCl, Cld1p was extracted more readily from mitochondria lacking CL, suggesting that CL facilitates Cld1p's membrane association. However, when analyzed by carbonate extraction, the extraction profile of Cld1p was similar in both wild type and Δ*crd1* mitochondria, except at pH 10.5, where Cld1p was slightly more extractable in the absence of CL (Figure 2.3A and B). Thus, Cld1p still acts as an interfacial membrane protein even in the absence of CL. Taken together, these data indicate that Cld1p bound to the IM via residues that extend into, but not through, the membrane independent of CL, but that CL contributes to the electrostatic interaction of Cld1p with the IM (Figure 2.3G).

The catalytic triad of Cld1p

Two motifs were predicted to be involved in Cld1p function (Beranek et al., 2009): the AXSXG motif, of which the Ser residue is expected to be critical for lipase or acyltransferase activity, and the HX_4D motif, a conserved structural motif in acyltransferases (Figure 2.4A). However, the requirement for either of these motifs has not been formally tested. Sequence analysis of Cld1p predicts that the C-terminus

contains an α/β hydrolase fold (Beranek et al., 2009), a family of enzymes with diverse substrate specificity but that invariably contain a catalytic triad consisting of a nucleophile, an acidic residue, and a histidine (Holmquist, 2000).

To identify the catalytic residues, a homology model of the α/β hydrolase domain was generated (Figure 2.4B) using the α/β hydrolase domain-containing CumD as a template (Fushinobu et al., 2002). The predicted nucleophile, Ser230, of the AXSXG motif, and residues within the HX_4D motif were individually mutated, as well as additional potential catalytic residues identified by their location within the homology model (Figure 2.4A and B). Each Cld1p mutant was expressed after being transformed into Δ*cld1*Δ*taz1* yeast, and its function analyzed by measuring the accumulation of MLCL (Figure 2.4C and D). Mutating Ser230 or His424 to Ala inactivated Cld1p. His424 is predicted to be adjacent to the catalytic Ser residue, consistent with its participation in catalysis. In contrast, mutating Asp429 within the HX_4D motif did not ablate Cld1p function, indicating that this motif may not be a *bona fide* transacylase motif. Instead, mutating Asp392, one of four additional Asp residues adjacent to the catalytic pocket identified in the homology model, to Asn abolished Cld1p activity. Importantly, mutating Ser230, His424, or Asp429 did not affect Cld1p assembly (Figure 2.5A), suggesting that the lack of Cld1p activity is not due to overall protein misfolding. Thus, Ser230, His424, and Asp392 compose the catalytic triad of Cld1p.

Cld1p functions as a monomer

The α/β hydrolase fold family of proteins contains enzymes that exist as monomers or higher oligomers (Carr and Ollis, 2009; Kim et al., 1997; Thoms et al., 2011). Analysis by 2D blue native/SDS-PAGE reveals that the majority of Cld1p

migrates between 80-120 kDa, but higher molecular weight complexes are also present (Figure 2.5A), suggesting dimerization or higher oligomerization. We sought to determine if this potential oligomerization is required for Cld1p activity by testing if a Cld1p catalytic mutant functions as a dominant negative. When expressed in a Δ*taz1* strain (which contains endogenous Cld1p), a dominant negative allele should inhibit the function of endogenous Cld1p, resulting in decreased MLCL and increased CL compared to Δ*taz1*. Wild type or the H424A Cld1p mutant were expressed from low (centromeric, ↑) or high (2 μm, ↑↑) copy plasmids in a Δ*taz1* strain (Figure 2.5B). However, phospholipid analysis showed no difference in the levels of CL and MLCL compared to Δ*taz1* rescued with empty vector (EV; Figure 2.5C). Therefore, despite its participation in higher-order assemblies (either with itself, other proteins, or phospholipids), oligomerization is not required for Cld1p function indicating that the functional unit is likely a monomer.

Cld1p is upregulated during respiratory conditions

The regulation of CL remodeling has not been documented. To this end, the steady state expression of Cld1p and Taz1p, as well as CL and MLCL levels, were examined in yeast grown in various carbon sources. In dextrose, which represses proteins involved in mitochondrial-mediated metabolism (Ohlmeier et al., 2004), Cld1p was barely detectable, whereas in raffinose, a fermentable carbon source that does not result in glucose repression, Cld1p was expressed at higher levels. Cld1p and Taz1p expression was highest when grown in the non-fermentable carbon source, lactate (Figure 2.6A and B). Importantly, no differences in expression were observed between wild type, Δ*cld1*, and Δ*taz1* yeast grown in the same media (Figure 2.7), indicating that the

presence of a functional remodeling pathway does not regulate the expression of Cld1p or Taz1p.

Analysis of mitochondrial phospholipids revealed that the sum of CL + MLCL, a gauge of CL biosynthesis that is equivalent for wild type, Δ*cld1*, and Δ*taz1* mutants, is reduced in yeast grown in dextrose compared to raffinose or rich lactate (Figure 2.6C, D and 8), consistent with previous studies of CL biosynthesis (Chen et al., 2008; Claypool et al., 2008a; Gu et al., 2004; Shen and Dowhan, 1998; Su and Dowhan, 2006). Similar to the steady state expression, no differences are observed in CL + MLCL levels between wild type, Δ*cld1*, or Δ*taz1* yeast grown in the same media (Figure 2.6D), demonstrating that CL biosynthesis is not affected by defects in the CL remodeling pathway.

In Δ*taz1* yeast, the ratio of the accumulated MLCL to CL provides a means to determine the function of Cld1p. In the absence of Taz1p MLCL, generated by Cld1p, cannot be reacylated and thus accumulates. The MLCL:CL ratio in Δ*taz1* yeast, and therefore the function of Cld1p, is lowest in the presence of dextrose, and highest in the presence of lactate (Figure 2.6E), correlating with the steady state expression levels of Cld1p (Fig 6B).

To further examine how Cld1p expression levels affect its function, Cld1p was overexpressed in Δ*taz1* yeast. Expression of *CLD1* from a centromeric plasmid resulted in similar increases in Cld1p steady state expression compared to endogenous Cld1p in each media type (2.6-fold higher in both dextrose and raffinose, 2.9-fold higher in lactate; Figure 2.9A and B). Overexpression of Cld1p did not alter the total amount of CL + MLCL (Figure 2.9C and D), although in lactate-containing media CL was significantly decreased (Figure 2.10A), and in raffinose-containing media MLCL was significantly

increased (Figure 2.10B). In all media types, the MLCL:CL ratio was slightly but significantly increased when Cld1p was overexpressed (Figure 2.9E), although to a lesser extent than expected based on the degree of overexpression (compare Δ*taz1* [EV] in rich lactate and Δ*taz1* [*CLD1*] in raffinose), which suggests that factors other than simply steady state abundance regulate Cld1p function.

Cld1p function is enhanced after dissipation of the mitochondrial membrane potential

To gain further insight into the regulation of CL remodeling, we examined the CL and MLCL levels in yeast treated with mitochondrial ionophores. CL biosynthesis has been shown to decrease when the mitochondrial pH gradient is dissipated with carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), but not when the membrane potential is ablated with valinomycin (Gohil et al., 2004); however the role of the electrochemical gradient in regulating CL remodeling has never been tested. 20 μM CCCP and 1 μM valinomycin inhibited growth in lactate-containing media, but displayed no effect on cell growth in media containing raffinose as the carbon source (Figure 2.11A and B). Steady state levels of mitochondrial phospholipids were analyzed after treatment for 24 hours in raffinose-containing media (Figure 2.12A and B). The levels of CL + MLCL in wild type yeast were not significantly affected by treatment with either CCCP or valinomycin, demonstrating that CL biosynthesis is not affected by chronic disruption of the mitochondrial membrane potential or pH gradient. Treatment with CCCP or valinomycin did not affect the total levels of CL + MLCL in either mutant, indicating that defects in the remodeling pathway compounded with sustained dissipation of the electrochemical gradient do not affect CL biosynthesis. The MLCL:CL ratio in Δ*taz1* yeast increased

after treatment with either CCCP or valinomycin (Figure 2.12C), signifying that Cld1p function is enhanced in the absence of a membrane potential. This increase in function is not due to an increase in Cld1p expression (Figure 2.12D and E), suggesting that the increased MLCL:CL ratio is due to increased Cld1p activity. Of note, Taz1p expression did increase after treatment with the ionophores (Figure 2.11C), indicating that regulation of its expression is independent of Cld1p expression.

DISCUSSION

A new step required for CL remodeling

While much is known about the enzymatic reactions involved in the biosynthesis and remodeling of CL, relatively little is known about CL trafficking. Namely, how does immature CL synthesized on the matrix-facing leaflet of the IM gain access to tafazzin on the IMS-facing leaflets of the inner and outer membranes to complete CL remodeling? To begin to answer this question, we determined the submitochondrial localization and membrane association of Cld1p, the lipase which initiates CL remodeling. Here, we have shown that Cld1p is embedded in the IM, but does not span through it to the IMS. Because Cld1p in Δ*crd1* mitochondria remained membrane bound in the absence of added salt after sonication, and its carbonate extraction profile did not change at a higher pH compared to wild type, it is unlikely that Cld1p is simply tethered to the membrane by CL. Rather, that Cld1p is more readily extracted by salt in the absence of CL suggests that its electrostatic interaction with the membrane is, at least partially, via the negatively charged headgroup of its substrate, CL.

The nature of Cld1p's membrane association is consistent with its function as a CL deacylase. Similar to the interfacial membrane protein Taz1p (Claypool et al., 2006), Cld1p must catalyze a reaction within the membrane bilayer, at the interface between the hydrophilic headgroups and hydrophobic fatty acyl chains. This is in contrast to an enzyme that catalyzes a reaction on the headgroup of a phospholipid such as Gep4p, which dephosphorylates phosphatidylglycerolphosphate to phosphatidylglycerol and is only peripherally attached to the IM (Osman et al., 2010).

The enzymatic activity of Crd1p (Schlame and Haldar, 1993) also occurs on the matrix-facing leaflet of the IM, suggesting that after synthesis, CL is deacylated by Cld1p forming MLCL, and that MLCL is the phospholipid that must be transported across the IM to Taz1p-containing membranes to complete CL remodeling (Figure 2.3G). Since Cld1p does not span the IM, its enzymatic activity is not coupled to the flipping of MLCL to the IMS-facing leaflet of the IM. The generation of mature CL requires multiple rounds of deacylation and reacylation. Consequently, after MLCL is translocated to Taz1p-containing leaflets for reacylation, newly reacylated CL may need to traffic back to the matrix-facing leaflet of the IM to become available for Cld1p to remove another acyl chain; a cycle that would repeat until mature CL is generated. However, Taz1p-mediated transacylation requires only a phospholipid and a lysophospholipid (Malhotra et al., 2009) in the context of a curved membrane (Schlame et al., 2012). Thus, it is possible that the lysophospholipid generated after the first reacylation of CL is used as an acyl chain acceptor for Taz1p to subsequently re-derive MLCL from CL. Thus, after Cld1p initiates CL remodeling by generating MLCL on the matrix-facing leaflet of the IM, the subsequent acylation/deacylation reactions would be mediated solely by Taz1p via transacylation and occur within the same leaflet. In either scenario, after Cld1p initiates CL remodeling by generating MLCL, an as yet unidentified protein(s) must mediate the redistribution of MLCL to the IMS-facing leaflets of the inner and outer membranes.

Is/are there any attractive candidate(s) for this novel activity? The simple answer is no. While proteins mediating phospholipid redistribution between membrane leaflets have been identified for the plasma membrane, ER, Golgi, and endosomes (van Meer et

al., 2008), little is known about phospholipid trafficking in the mitochondrion. CL redistribution between IM leaflets has been demonstrated (Gallet et al., 1997; Gallet et al., 1999); however, the player(s) responsible have not been identified.

A novel link between CL remodeling and OXPHOS

CL biosynthesis is known to be modulated in yeast by the available carbon source; CL levels are decreased in dextrose and increased in non-fermentable carbon sources (Figure 2.6D) (Chen et al., 2008; Claypool et al., 2008a; Gu et al., 2004; Shen and Dowhan, 1998; Su and Dowhan, 2006). Additionally, decreasing the matrix pH reduces Crd1p function, in turn decreasing the steady state levels of CL (Gohil et al., 2004). Here, we show that the sum of CL + MLCL, an indicator of CL biosynthesis, is decreased in dextrose compared to raffinose and lactate. Importantly, no difference in CL + MLCL levels are observed between wild type, Δ*cld1*, and Δ*taz1* yeast, indicating that the differences observed between the various carbon sources are the result of regulation at the level of CL biosynthesis, not remodeling.

In contrast, nothing is known about the regulation of CL remodeling. To investigate this important issue, we addressed three specific questions: 1) Is the CL remodeling pathway regulated?; 2) If yes, is there a particular step in the pathway that serves as the master regulator?; and 3) Is CL remodeling regulated in a similar or distinct way as CL biosynthesis?

Cld1p and Taz1p expression is increased by approximately two fold in lactate compared to raffinose, despite no difference in CL + MLCL levels. Cld1p, but not Taz1p, expression is repressed in dextrose, consistent with the reduced steady state levels of CL + MLCL. These observations indicate that Cld1p expression is not exclusively

coordinated by CL steady state levels. More importantly, they indicate that the abundance of CL remodeling enzymes is regulated by available carbon source.

The MLCL:CL ratio in Δ*taz1* yeast, a measure of Cld1p function, is decreased in dextrose and increased in lactate, correlating with the steady state expression level of Cld1p. By itself, this data suggests that Cld1p function is regulated by the steady state abundance of the Cld1p polypeptide. However, overexpression of Cld1p did not result in a proportional increase in the MLCL:CL ratio, suggesting additional modes of Cld1p regulation.

While the presence of various carbon sources and the treatment with ionophores modulates CL remodeling, neither alteration resulted in the accumulation of MLCL in wild type compared to Δ*cld1*. Thus, the activity of both Taz1p and the unidentified MLCL trafficking protein(s) were not limiting. Of note, Taz1p expression increased after treatment with valinomycin (Figure 2.11C), suggesting that its expression is modulated to match the rate of CL remodeling. Further, Taz1p expression is not increased due to an increase in its substrate MLCL, since similar increases in Taz1p expression occur in both wild type and Δ*cld1* after treatment with valinomycin. In sum, these results indicate that removal of an acyl chain from CL by Cld1p represents the major site of regulation for the CL remodeling pathway.

CCCP, a H^+ ionophore, dissipates the mitochondrial membrane potential as well as decreases the matrix pH, whereas valinomycin, a K^+ ionophore, decreases the membrane potential while maintaining the matrix pH (Gohil et al., 2004). Treatment of yeast for 24 hours with a non-lethal concentration of either ionophore did not result in a significant alteration of CL + MLCL levels (Figure 2.12B, the observed decrease was not

statistically significant). This is in contrast to another study that reported a decrease in CL synthesis after treatment with CCCP (Gohil et al., 2004), although in these experiments phospholipids were pulse labeled and yeast were treated with ionophores for a shorter time period. In Δ*taz1* yeast, the MLCL:CL ratio increased after treatment with either CCCP or valinomycin, despite unchanged expression of Cld1p. Both ionophores decrease the membrane potential, but only CCCP affects the matrix pH, thus Cld1p function increases as the membrane potential decreases.

Taken together, these data indicate that CL remodeling can be regulated by two different mechanisms. First, Cld1p expression (and thus overall function) is regulated by the available carbon source, similar to the regulation of CL biosynthesis (Chen et al., 2008; Claypool et al., 2008a; Gu et al., 2004; Shen and Dowhan, 1998; Su and Dowhan, 2006), which may allow CL biosynthesis and remodeling rates to be coordinated. Second, Cld1p function is regulated by changes in the mitochondrial membrane potential, representing a regulatory mechanism that is distinct from that of Crd1p (Gohil et al., 2004), and suggests that a cell can individually fine tune both the total levels of CL and the molecular form of CL.

There are at least two non-mutually exclusive benefits of having flux through the CL remodeling pathway regulated by the strength of the membrane potential across the IM (Figure 2.13). First, physiologically, decreases in the mitochondrial membrane potential indicate greater energetic demand (Huttemann et al., 2008). Essentially, this is a simple way for the cell to coordinate energy production with energy demand. For instance, when cytosolic ATP is depleted, the relative concentration of ADP increases; condensation of ADP and P_i by the ATP synthase is coupled to the down-hill flow of

protons across the IM through the F_o section which dissipates the membrane potential (the process of respiratory control) (Chance and Williams, 1955). CL remodeling has been proposed to generate a form of CL that functions more optimally than newly synthesized CL (Cheng et al., 2008; Claypool and Koehler, 2012; Schlame et al., 2005). If true, then by augmenting Cld1p activity upon dissipation of the membrane potential the relative abundance of remodeled CL would increase, in turn promoting the enhanced activity of the OXPHOS machinery. This novel potential feedback mechanism would therefore link energetic demand with the capacity to produce energy by altering the acyl chain composition of CL.

Second, mitochondria are the major producers of cellular reactive oxygen species (ROS). CL is intimately associated with all of the main players involved in OXPHOS and is susceptible to oxidation (Kim et al., 2011). Further, peroxidized CL inactivates complex IV (Musatov, 2006). Defects in the respiratory chain, either from mutation or through pharmacological insult, have been shown to increase ROS production and decrease the membrane potential (Baile and Claypool, 2013; Grivennikova and Vinogradov, 2006; Minners et al., 2007; Quinlan et al., 2011). Therefore, CL remodeling could be activated as part of a mitochondrial ripple-response cascade (Vafai and Mootha, 2012), maintaining OXPHOS capacity by repairing damaged CL molecules. The dissipation of the membrane potential would act as a signal to activate CL remodeling, removing oxidatively damaged acyl chains from CL by Cld1p and replacing them with unadulterated acyl chains by tafazzin-mediated transacylation. In this scenario, OXPHOS capacity would be maintained but not necessarily enhanced by fixing damaged CL molecules, providing a mechanism whereby the membrane potential is reestablished.

This in turn would prevent more drastic downstream consequences associated with severe reductions in the membrane potential including mitophagy or apoptosis (Clayton et al., 2005; Frezza et al., 2006; Jin et al., 2010; Twig et al., 2008). Consistent with this model, oxidative damage is increased in the absence of tafazzin in yeast (Chen et al., 2008) and humans (Gonzalvez et al., 2013), implicating CL remodeling in reducing ROS formation.

MATERIALS AND METHODS

Yeast strains and growth conditions

All strains were derived from the wild type parental *S. cerevisiae* strain GA74-1A (*MAT**a**, his3-11,15, leu2, ura3, trp1, ade8, rho*$^{+}$*, mit*$^{+}$). Δ*crd1* (*MAT**a**, his3-11,15, leu2, ura3, ade8,* Δ*crd1*::*TRP1*) has been described previously (Claypool et al., 2008b). Δ*cld1* (*MAT**a**, leu2, ura3, trp1, ade8,* Δ*cld1*::*HISMX6*), Δ*taz1* (*MAT**a**, his3-11,15, leu2, trp1, ade8,* Δ*taz1*::*URAMX*), Δ*cld1*Δ*taz1* (*MAT**a**, leu2, trp1, ade8,* Δ*cld1*::*HISMX6,* Δ*taz1*::*URAMX*), Δ*cox4* (*MAT**a**, his3-11,15, leu2, ura3, ade8* Δ*cox4*::*TRP1*), Δ*rip1* (*MAT**a**, leu2, ura3, trp1, ade8,* Δ*rip1*::*HIS3MX6*), and Δ*qcr6* (*MAT**a**, his3-11,15, leu2, ura3, ade8* Δ*qcr6*::*TRP1*) were generated by replacing the entire open reading frame of the gene using PCR-mediated gene replacement (Wach et al., 1994).

Yeast were grown in YP media (1% yeast extract, 2% peptone) supplemented with either 2% dextrose or 2% raffinose in figures 2.6 and 2.8; synthetic drop out media (0.17% yeast nitrogen base, 0.5% ammonium sulfate, 0.2% drop out mix synthetic –leu) supplemented with 2% dextrose or 2% raffinose in figure 2.9; synthetic rich lactate –leu (0.17% yeast nitrogen base minus amino acids and ammonium sulfate, 0.5% ammonium sulfate, 0.2% dropout mix synthetic –leu, 0.05% dextrose, 2% lactic acid, 3.4 mM $CaCl_2$-$2H_2O$, 8.5 mM NaCl, 2.95 mM $MgCl_2$-$6H_2O$, 7.35 mM KH_2PO_4, 18.7 mM NH_4Cl, pH 5.5) in figures 2.2, 2.4, 2.5, and 2.7; and rich lactate media (1% yeast extract, 2% tryptone, 0.05% dextrose, 2% lactic acid, 3.4 mM $CaCl_2$-$2H_2O$, 8.5 mM NaCl, 2.95 mM $MgCl_2$-$6H_2O$, 7.35 mM KH_2PO_4, 18.7 mM NH_4Cl, pH 5.5) in all other experiments.

For treatment with ionophores, overnight cultures grown in YP-raffinose or rich lactate were diluted to 0.4 OD_{600}/ml in YP-raffinose or rich lactate containing 20 μM

CCCP, 1 μM valinomycin, or an equal volume of vehicle (ethanol for CCCP, DMSO for valinomycin). For growth analysis, the OD_{600} was measured at 0, 2, 4, 6, 8, and 24 hrs. Phospholipid steady state levels and protein expression was analyzed after 24 hrs of treatment.

Molecular Biology

CLD1 was amplified from genomic DNA isolated from GA74-1A yeast using primers that hybridized ~450 bp 5' of the predicted start codon and ~100 bp 3' of the predicted stop codon and subcloned into pRS315. *CLD1* point mutations and both the mature N- and C-terminal CNAP tagged *CLD1* were generated by overlap extension (Ho et al., 1989) using pRS315CLD1 as a template.

Digitonin based submitochondrial localization

Performed as described previously (Glick et al., 1992; Tamura et al., 2012). Briefly, 250 μg mitochondria were resuspended in SEHK buffer (250 mM sucrose, 5 mM EDTA, 10 mM HEPES-KOH pH7.4, 50 mM KCl, 200 μM PMSF) supplemented with 0-0.5% (w/v) digitonin and solubilized for 60 sec. 8.5 volumes cold SEHK buffer was added to stop solubilization. Released proteins were separated from the membrane pellet by centrifugation at 100,000 x *g* (TLA120.1 rotor) for 10 min at 4°C. The supernatant fraction was TCA precipitated, and both supernatant and membrane pellet fractions resuspended in equal volumes of sample buffer. After separation by SDS-PAGE and immunoblotting as indicated, band intensities were captured with the Fluorchem Q (Cell Biosciences, Santa Clara, CA) quantitative digital imaging system and quantified using affiliated AlphaView SA.

Sonication

500 µg of mitochondria were osmotically shocked by resuspending in mitoplasting buffer (30 mM Sorbitol, 20 mM HEPES, pH 7.4) and incubating on ice for 30 min. IMS proteins were released by adding KCl in mitoplasting buffer to a final concentration of 0-1M and incubation on ice for 15 min. Released proteins (IMS fraction) were separated from mitoplasts by centrifugation at 21,000 x *g* for 10 min at 4°C and TCA precipitated. Mitoplast pellets were resuspended to 10 mg/ml in sonication buffer (0.6 M Sucrose, 3 mM $MgCl_2$, 20 mM HEPES, pH 7.4) containing 0-1M KCl, and sonicated 3 x 10 sec with 30 sec intervals using a Sonic Dismembrator 500 (Fisher Scientific, Lafayette, CO) at amplitude 15%. After removal of unbroken mitoplasts by centrifugation, the supernatant was separated from the membrane pellet by centrifugation at 100,000 x *g* for 30 min at 4°C (TLA120.1 rotor), and the supernatant TCA precipitated. Equal volumes of each fraction were resolved by SDS-PAGE and immunoblotted. When the IMS fraction was not analyzed, 500 µg of mitochondria were collected by centrifugation, resuspended to 10 mg/ml in sonication buffer, and treated as described above.

Antibodies

Most antibodies used in this study were generated in the Schatz (J. Schatz, Univeristy of Basel, Basel, Switzerland) or Koehler (C. Koehler, University of California, Los Angeles, Los Angeles, CA) laboratories and have been described previously (Claypool et al., 2008a; Claypool et al., 2006; Claypool et al., 2011; Daum et al., 1982; Hwang et al., 2007; Riezman et al., 1983; Whited et al., 2012). Antibodies against Cld1p, Cox4p, Rip1p, and Qcr6p were raised in rabbits using purified recombinant proteins as antigens. The specificity of each antibody is provided in Figure 2.14. Recombinant

proteins were generated essentially as described (Claypool et al., 2006; Claypool et al., 2011), by cloning the entire open reading frame into the pET28a vector (Novagen, Darmstadt, Germany), downstream of the His_6 tag, induced in BL21-CodonPlus(DE3)-RIL *Escherichia coli*, and purified using Ni2+ agarose (Qiagen, Valencia, CA). Other antibodies used were mouse anti-Sec62p (gift from D. Meyers, University of California, Los Angeles, Los Angeles, CA), mouse anti-Aac2p clone 6H8 (Panneels et al., 2003), mouse anti-Protein C (Roche, Indianapolis, IN) and horseradish peroxidase-conjugated (Thermo Fisher Scientific, Lafayette, CO) or fluorescent-conjugated (Pierce, Rockford, IL) secondary antibodies.

Miscellaneous

Isolation of mitochondria, subcellular fractionation, preparation of yeast cell extracts, 2D blue native/SDS-PAGE, phospholipid analyses, and immunoblotting were performed as previously described (Claypool et al., 2008a; Claypool et al., 2006). Carbonate extraction was performed as previously described (Claypool et al., 2006), except that the pellet and supernatant fractions were separated by centrifugation at 175,000 x *g* for 15 min at 4°C using a TLA120.1 rotor. The proteinase K accessibility assay was performed as previously described (Claypool et al., 2006), except that 0.5% (w/v) deoxycholate was used to solubilize the IM. Phosphate quantification was performed as described (Rouser et al., 1970). The homology model of the α/β hydrolase domain of Cld1p was generated using the SWISS-MODEL Workspace (Arnold et al., 2006; Guex and Peitsch, 1997; Schwede et al., 2003). The predicted mature N-terminus of Cld1p was determined using MitoProt II v1.101 *(Claros and Vincens, 1996).* Statistical comparisons were performed by *t*-test or one way analysis of variance

(ANOVA) with Holm-Sidak pairwise comparison using SigmaPlot 11 software (Systat Software, San Jose, CA).

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TABLES

Table 2.1. Cld1p transmembrane domain predictions

TM prediction provider	Amino acids of predicted TM domains	Reference
DAS-TMfilter prediction server http://www.enzim.hu/DAS/DAS.html	None predicted	(Cserzo et al., 2004)
TMpred http://www.ch.embnet.org/software/TMPRED_form.html	142-160, 225-241	(Hofmann and Stoffel, 1993)
HMMTOP http://www.enzim.hu/hmmtop/	143-160	(Tusnady and Simon, 1998)
TMHMM Server v.2.0 http://www.cbs.dtu.dk/services/TMHMM/	None predicted	(Krogh et al., 2001)
SPLIT http://split.pmfst.hr/split/4/	None predicted	(Juretic et al., 2002)

FIGURES

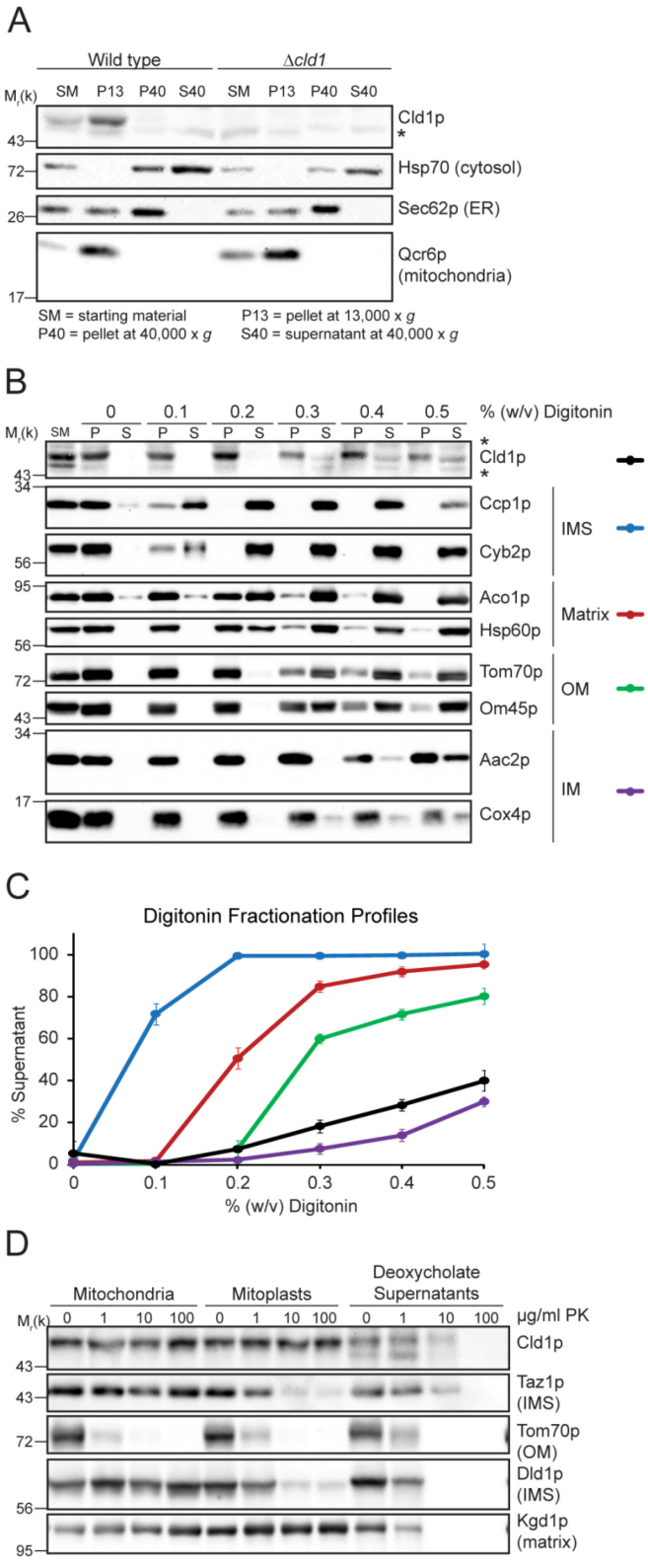
A
Wild type
Δcld1
M_r(k)
SM P13 P40 S40 SM P13 P40 S40
Cld1p
*
Hsp70 (cytosol)
Sec62p (ER)
Qcr6p
(mitochondria)
43
72
26
17
SM = starting material
P40 = pellet at 40,000 x g
P13 = pellet at 13,000 x g
S40 = supernatant at 40,000 x g
B
0 0.1 0.2 0.3 0.4 0.5 % (w/v) Digitonin
M_r(k) SM P S P S P S P S P S P S
Cld1p
Ccp1p
Cyb2p
Aco1p
Hsp60p
Tom70p
Om45p
Aac2p
Cox4p
IMS
Matrix
OM
IM
C
Digitonin Fractionation Profiles
% Supernatant
% (w/v) Digitonin
D
Mitochondria
Mitoplasts
Deoxycholate Supernatants
M_r(k) 0 1 10 100 0 1 10 100 0 1 10 100
μg/ml PK
Cld1p
Taz1p
(IMS)
Tom70p
(OM)
Dld1p
(IMS)
Kgd1p
(matrix)

Figure 2.1. Cld1p resides in the mitochondrial inner membrane. (A) Yeast subcellular fractions were prepared by differential centrifugation. 50 µg (for Cld1p) or 25 µg (for all other proteins) of each fraction was separated by SDS-PAGE and immunoblotted as indicated. (B and C) Mitochondria isolated from wild type yeast were solubilized with the indicated concentration of digitonin. (B) Equal volumes of extracted (S) and non-extracted (P) protein for each digitonin concentration were separated by SDS-PAGE and immunoblotted. (C) The band intensities for two markers per compartment were combined and plotted as the percent of signal in the supernatant (mean ± SEM; *n*=3). (D) Intact mitochondria, mitoplasts, or deoxycholate solubilized mitochondria from wild type yeast were incubated with the indicated concentrations of proteinase K (PK). 50 µg of each sample were separated by SDS-PAGE and immunoblotted as indicated. *indicates a non-specific cross reaction of the Cld1p antiserum.

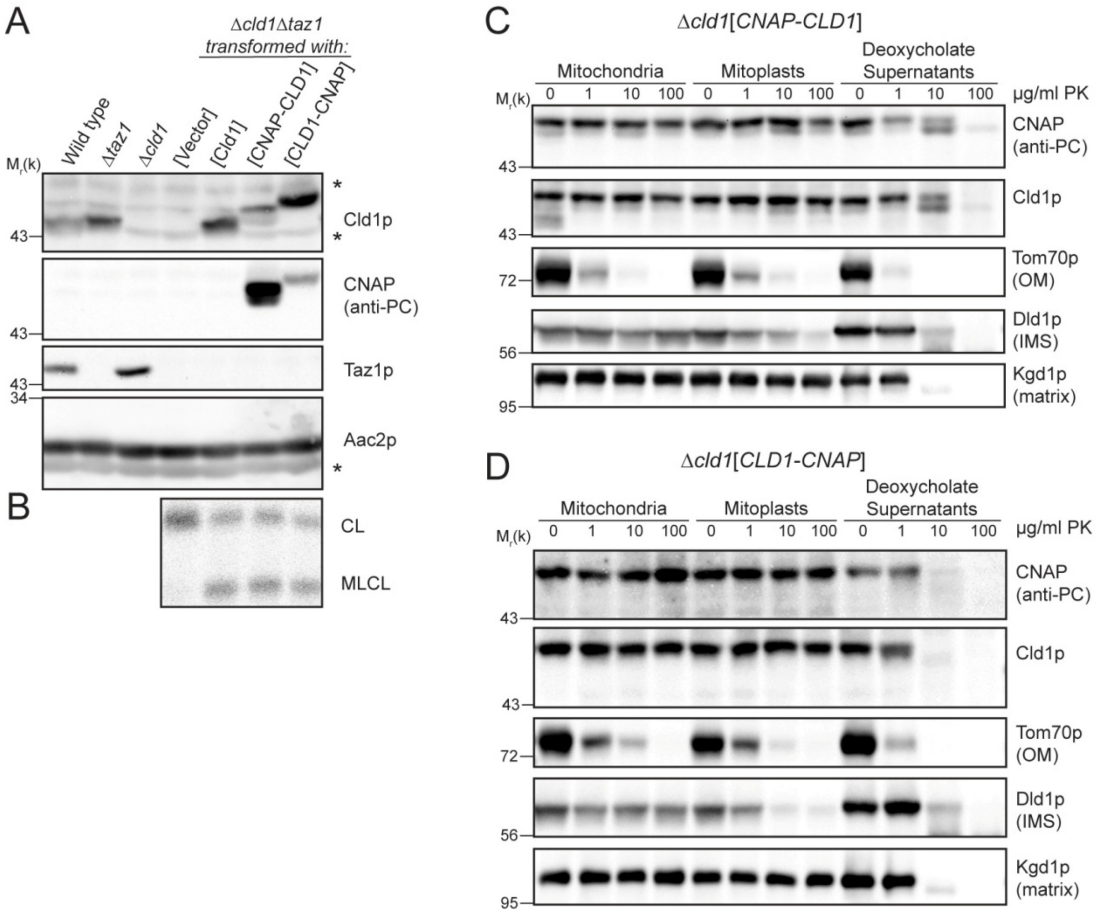

Figure 2.2. Both termini of Cld1p face the matrix. (A) Whole cell extracts were separated by SDS-PAGE and immunoblotted as indicated. *indicates a non-specific cross reaction of the Cld1p antiserum. (B) Mitochondrial phospholipids from the indicated strains were labeled with $^{32}P_i$ and separated by TLC. (C and D) Intact mitochondria, mitoplasts, or deoxycholate solubilized mitochondria from Δ*cld1* yeast transformed with (C) CNAP-Cld1p or (D) Cld1p-CNAP were incubated with the indicated concentrations of proteinase K (PK). 50 μg of each sample were separated by SDS-PAGE and immunoblotted as indicated.

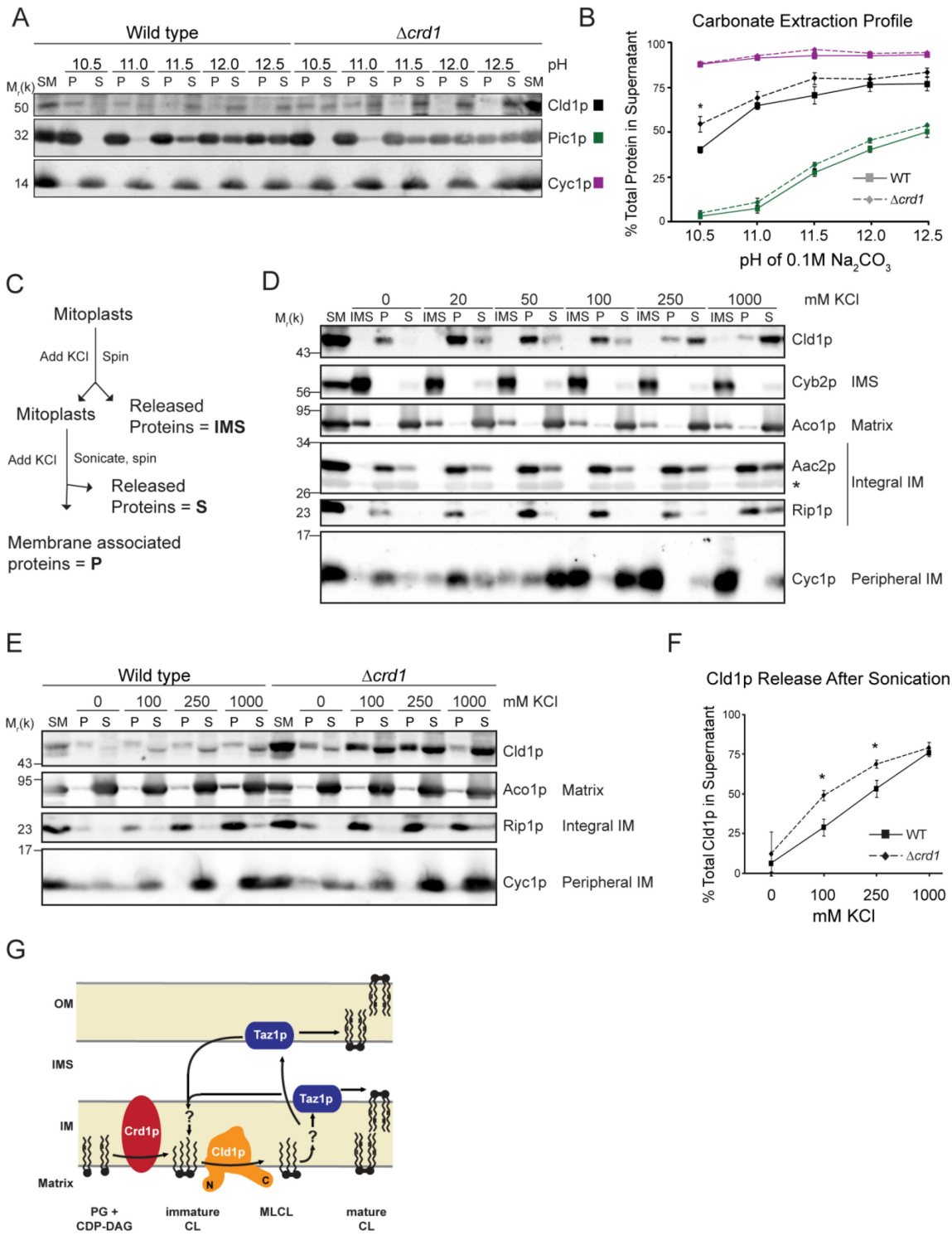
A
Wild type
Δcrd1
10.5 11.0 11.5 12.0 12.5 10.5 11.0 11.5 12.0 12.5
pH
M_r(k) SM P S P S P S P S P S P S P S P S P S P S SM
50
32
14
Cld1p
Pic1p
Cyc1p
B
Carbonate Extraction Profile
% Total Protein in Supernatant
100
75
50
25
0
WT
Δcrd1
10.5 11.0 11.5 12.0 12.5
pH of 0.1M Na2CO3
C
Mitoplasts
Add KCl
Spin
Mitoplasts
Released Proteins = IMS
Add KCl
Sonicate, spin
Released Proteins = S
Membrane associated proteins = P
D
0 20 50 100 250 1000 mM KCl
M_r(k) SM IMS P S IMS P S IMS P S IMS P S IMS P S IMS P S
43
56
95
34
26
23
17
Cld1p
Cyb2p IMS
Aco1p Matrix
Aac2p *
Rip1p
Integral IM
Cyc1p Peripheral IM
E
Wild type
Δcrd1
0 100 250 1000 0 100 250 1000 mM KCl
M_r(k) SM P S P S P S P S SM P S P S P S P S
43
95
23
17
Cld1p
Aco1p Matrix
Rip1p Integral IM
Cyc1p Peripheral IM
F
Cld1p Release After Sonication
% Total Cld1p in Supernatant
100
75
50
25
0
WT
Δcrd1
0 100 250 1000
mM KCl
G
OM
IMS
IM
Matrix
Taz1p
Taz1p
Crd1p
Cld1p
N
C
PG + CDP-DAG
immature CL
MLCL
mature CL

Figure 2.3. Cld1p is associated with the matrix-facing leaflet of the IM. (A) Wild type or Δ*crd1* mitochondria were incubated in 0.1M carbonate of the indicated pH. Membrane bound proteins (P) were separated from released proteins (S) by ultracentrifugation and equal volumes of each fraction were resolved by SDS-PAGE and immunoblotted as indicated. (B) Band intensities of the P and S fractions were quantified and plotted as the percentage of total protein released into the supernatant for each pH (mean ± SEM; n=4). Solid and dashed lines indicate wild type and Δ*crd1* mitochondria, respectively. (C) Outline of the sonication experiment in (D). (D) Wild type mitoplasts were incubated with the indicated concentration of KCl, and pelleted by centrifugation. Released proteins (IMS) in the supernatant were removed and TCA precipitated. Mitoplasts were resuspended in buffer maintaining the indicated KCl concentration and sonicated. Membranes (P) were separated from released proteins (S) by centrifugation. Equal amounts of each sample were resolved by SDS-PAGE and immunoblotted as indicated. (E) Intact wild type or Δ*crd1* mitochondria were sonicated in the presence of the indicated KCl concentrations as in (D). (F) Band intensities of the P and S fractions were quantified and plotted as the percentage of total protein released into the supernatant for each KCl concentration (mean ± SEM; n=4). (G) Cld1p is embedded in the IM facing the mitochondrial matrix. * in (B) and (F) indicates a statistically significant difference ($p < 0.05$) as determined by t-test.

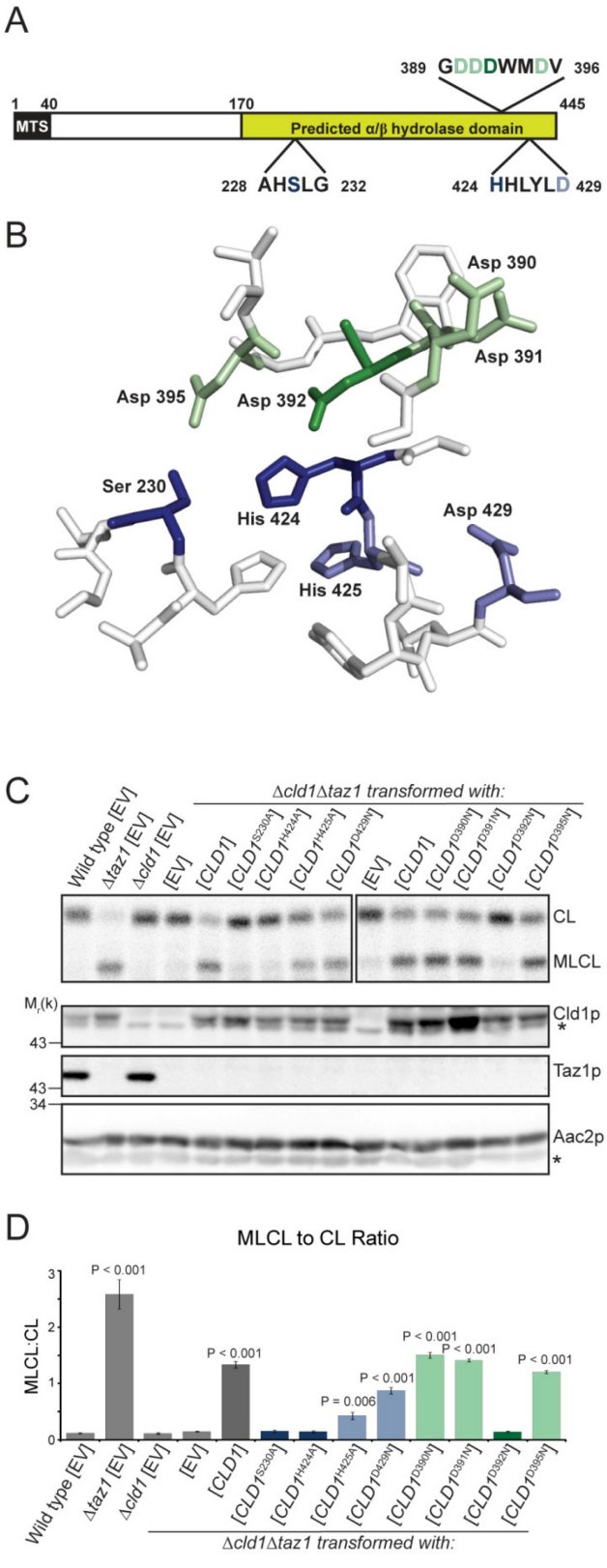
A
389 GDDDWMDV 396
1 40 170 445
MTS
Predicted α/β hydrolase domain
228 AHSLG 232
424 HHLYLD 429
B
Asp 390
Asp 391
Asp 395
Asp 392
Ser 230
His 424
Asp 429
His 425
C
Δcld1Δtaz1 transformed with:
Wild type [EV]
Δtaz1 [EV]
Δcld1 [EV]
[EV]
[CLD1]
[CLD1S230A]
[CLD1H424A]
[CLD1H425A]
[CLD1D429N]
[EV]
[CLD1]
[CLD1D390N]
[CLD1D391N]
[CLD1D392N]
[CLD1D395N]
CL
MLCL
Mr(k)
43
Cld1p
*
43
Taz1p
34
Aac2p
*
D
MLCL to CL Ratio
MLCL:CL
P < 0.001
P < 0.001
P < 0.001
P = 0.006
P < 0.001
P < 0.001
P < 0.001
P < 0.001
Wild type [EV]
Δtaz1 [EV]
Δcld1 [EV]
[EV]
[CLD1]
[CLD1S230A]
[CLD1H424A]
[CLD1H425A]
[CLD1D429N]
[CLD1D390N]
[CLD1D391N]
[CLD1D392N]
[CLD1D395N]
Δcld1Δtaz1 transformed with:

Figure 2.4. The catalytic triad of Cld1p. (A) Schematic of Cld1p, containing a predicted mitochondrial targeting sequence (MTS) and α/β hydrolase domain. Amino acid residues mutated in the predicted lipase motif (residues 228-232) and acyltransferase structural motif (residues 424-429) are shown in blue; additional mutated residues in green. (B) The α/β hydrolase domain of Cld1p was modeled with SWISS-MODEL. Residues constituting the predicted catalytic pocket of Cld1p are shown, and colored as in (A). (C) Mitochondrial phospholipids from the indicated strains were labeled with $^{32}P_i$ and separated by TLC (top panel). Whole cell extracts were immunoblotted as indicated (bottom panels). (D) Quantification of the MLCL:CL ratio (mean ± SEM; *n*=6). Significant differences compared to Δ*cld1*Δ*taz1*[EV] were determined by one-way ANOVA. EV = empty vector. *indicates non-specific bands.

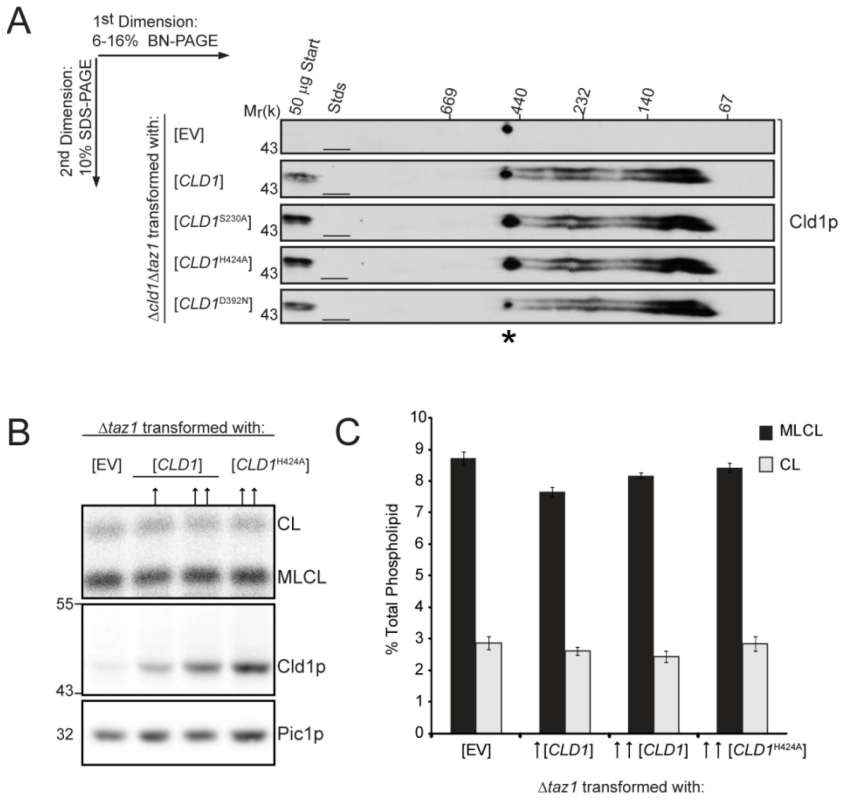

Figure 2.5. Cld1p functions as a monomer. (A) 150 µg mitochondria isolated from the indicated strains were solubilized with 1.5% (w/v) digitonin, separated by 2D blue native/SDS-PAGE, and immunoblotted for Cld1p. *indicates a non-specific cross reaction of the Cld1p antiserum. (B) Mitochondrial phospholipids from the indicated strains were labeled with $^{32}P_i$ and separated by TLC (top panel). Whole cell extracts from the indicated strains were resolved by SDS-PAGE and immunoblotted for Cld1p and the loading control Pic1p (bottom panels). ↑ indicates a low copy (centromeric) plasmid, ↑↑ indicates a high copy (2 µm) plasmid. (C) Quantification of CL and MLCL (mean ± SEM; *n*=6). EV = empty vector.

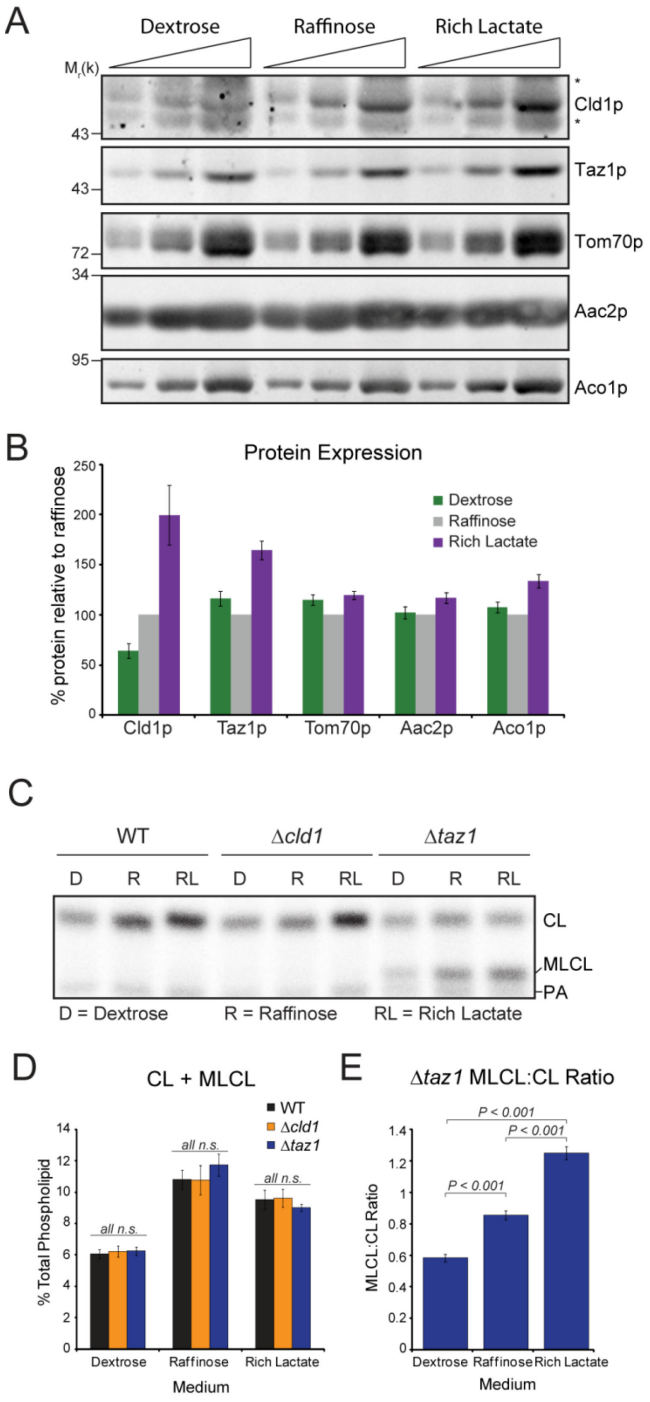
A
Dextrose
Raffinose
Rich Lactate
M_r(k)
43
43
72
34
95
Cld1p
Taz1p
Tom70p
Aac2p
Aco1p
B
Protein Expression
% protein relative to raffinose
250
200
150
100
50
0
Dextrose
Raffinose
Rich Lactate
Cld1p
Taz1p
Tom70p
Aac2p
Aco1p
C
WT
Δcld1
Δtaz1
D
R
RL
CL
MLCL
PA
D = Dextrose
R = Raffinose
RL = Rich Lactate
D
CL + MLCL
% Total Phospholipid
14
12
10
8
6
4
2
0
all n.s.
all n.s.
all n.s.
WT
Δcld1
Δtaz1
Dextrose
Raffinose
Rich Lactate
Medium
E
Δtaz1 MLCL:CL Ratio
MLCL:CL Ratio
1.4
1.2
1
0.8
0.6
0.4
0.2
0
P < 0.001
P < 0.001
P < 0.001
Dextrose
Raffinose
Rich Lactate
Medium

Figure 2.6. Cld1p expression and function is modulated by the available carbon source. (A) Whole cell extracts from wild type yeast grown in YP-dextrose (Dextrose), YP-raffinose (Raffinose), or rich lactate were resolved by SDS-PAGE and immunoblotted as indicated. *indicates non-specific cross reactions of the Cld1p antiserum. (B) Band intensities from wild type yeast were quantified and expressed as the % protein relative to raffinose (mean ± SEM, *n*=17). (C) Mitochondrial phospholipids from yeast grown in the indicated media were labeled with $^{32}P_i$ and separated by TLC. (D) The sum of CL + MLCL from the indicated strains (mean ± SEM, *n*=6). n.s. = differences not significant. (E) The ratio of MLCL:CL from Δ*taz1* (mean ± SEM, *n*=6). Significant differences determined by one-way ANOVA.

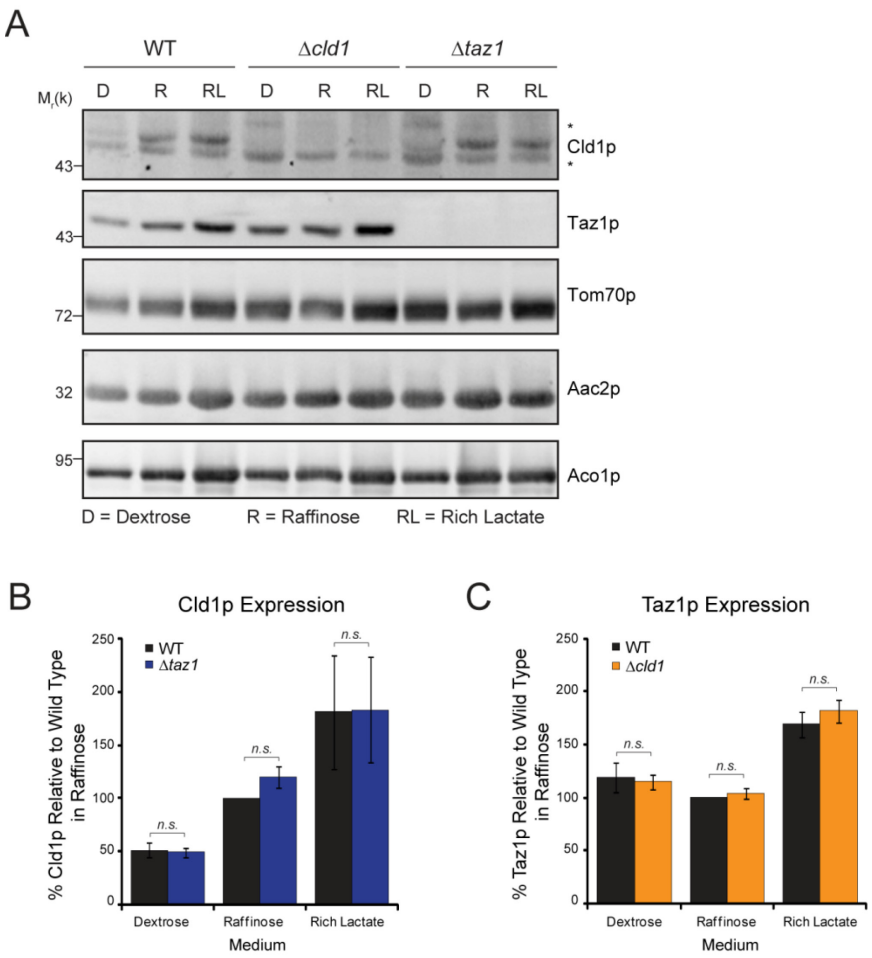

Figure 2.7. Cld1p and Taz1p expression is unaltered in CL remodeling pathway mutants. (A) Whole cell extracts from the indicated strains were grown in YP-dextrose (D), YP-raffinose (R), or rich lactate (RL), resolved by SDS-PAGE, and immunoblotted. (B) Cld1p or (C) Taz1p band intensities were quantified and plotted as the % protein relative to wild type grown in YP-raffinose (mean ± SEM, *n*=8). n.s. = differences not significant as determined by *t*-test

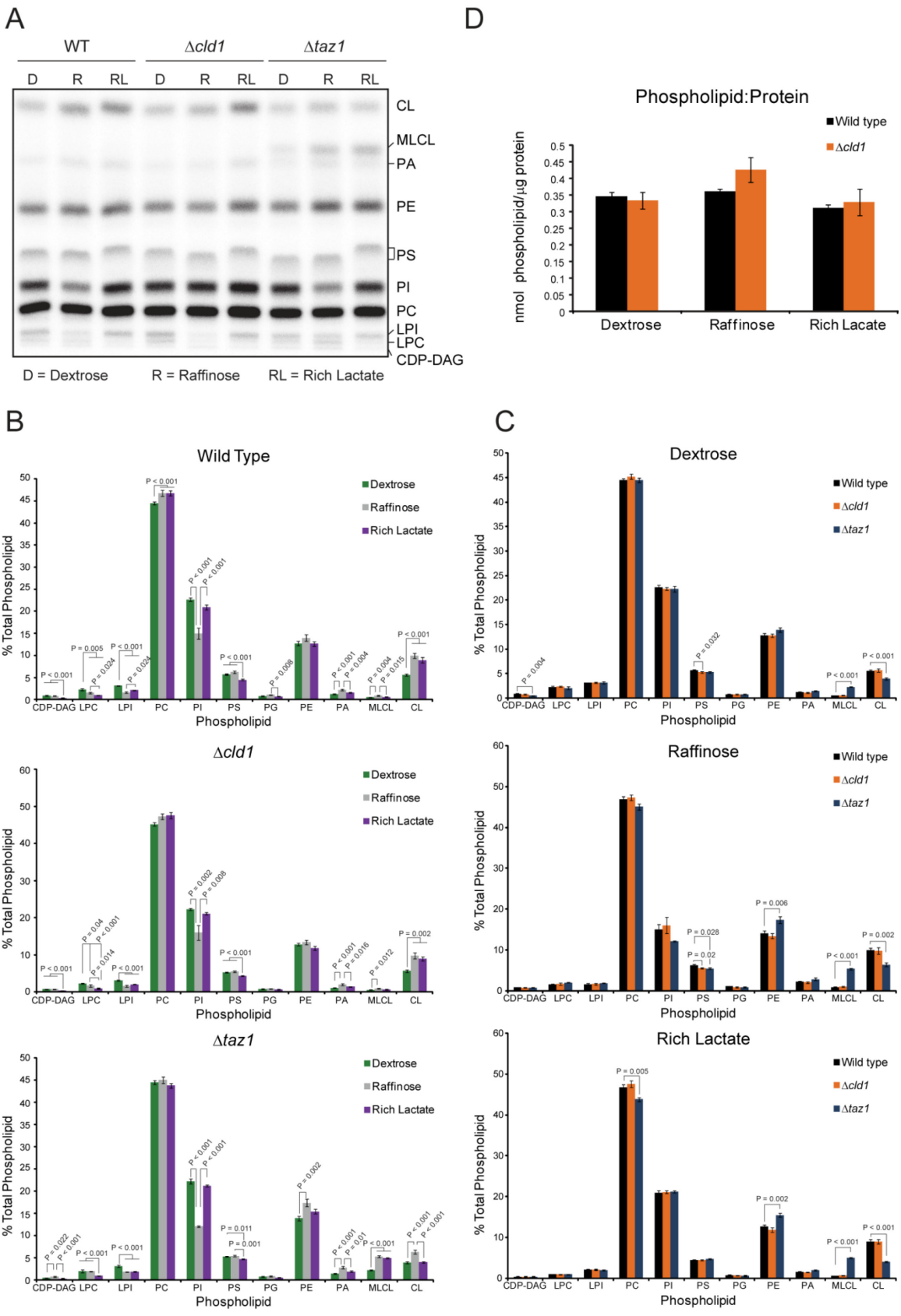
A
WT
Δcld1
Δtaz1
D
R
RL
CL
MLCL
PA
PE
PS
PI
PC
LPI
LPC
CDP-DAG
D = Dextrose
R = Raffinose
RL = Rich Lactate
D
Phospholipid:Protein
Wild type
Δcld1
nmol phospholipid/μg protein
Dextrose
Raffinose
Rich Lacate
B
Wild Type
Δcld1
Δtaz1
Dextrose
Raffinose
Rich Lactate
% Total Phospholipid
Phospholipid
CDP-DAG
LPC
LPI
PC
PI
PS
PG
PE
PA
MLCL
CL
C
Dextrose
Raffinose
Rich Lactate
Wild type
Δcld1
Δtaz1

Figure 2.8. The **effect of the available carbon source on mitochondrial phospholipids.** (A) The full TLC plate shown in figure 6C, showing the separation of mitochondrial phospholipids from yeast grown in the indicated media after $^{32}P_i$ labeling. (B and C) Quantification of mitochondrial phospholipids (mean ± SEM, *n*=8). (B) Comparison of mitochondrial phospholipids from the indicated strain grown in the presence of different carbon sources. (C) Comparison of mitochondrial phospholipids from various yeast strains grown in the indicated carbon source. Significant differences determined by one-way ANOVA with pairwise comparisons. (D) The ratio of phospholipid: protein in wild type and Δcld1 mitochondria (mean ± SEM, *n*=3). Differences are not significant as determined by *t*-test.

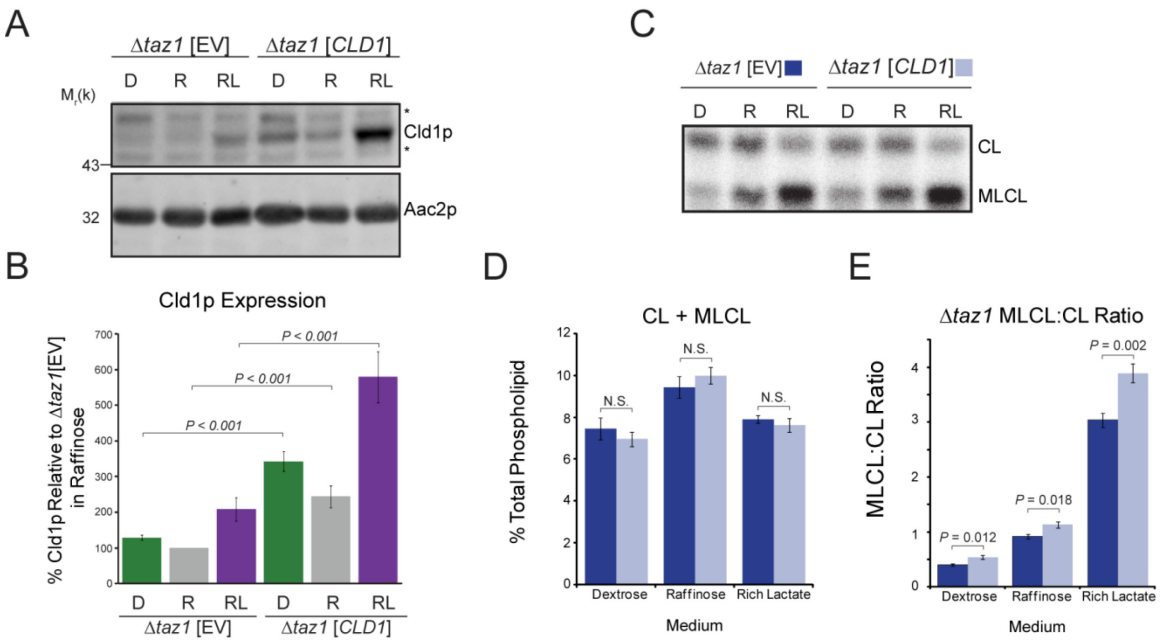

Figure 2.9. Cld1p overexpression does not cause a proportional increase in function. (A) Whole cell extracts from Δ*taz1* yeast transformed with an empty vector (EV) or *CLD1* grown in YP-dextrose (D), YP-raffinose (R), or rich lactate (RL) were separated by SDS-PAGE and immunoblotted as indicated. *indicates non-specific cross reactions of the Cld1p antiserum. (B) Cld1p band intensities were quantified and plotted as the % protein relative to Δ*taz1*[EV] grown in raffinose (mean ± SEM, *n*=8). (C) Mitochondrial phospholipids from yeast grown in the indicated media were labeled with $^{32}P_i$ and separated by TLC. (D) The sum of CL + MLCL from the indicated strains (mean ± SEM, *n*=6). n.s. = differences not significant. (E) The ratio of MLCL:CL from Δ*taz1* (mean ± SEM, *n*=6). Significant differences determined by *t*-test.

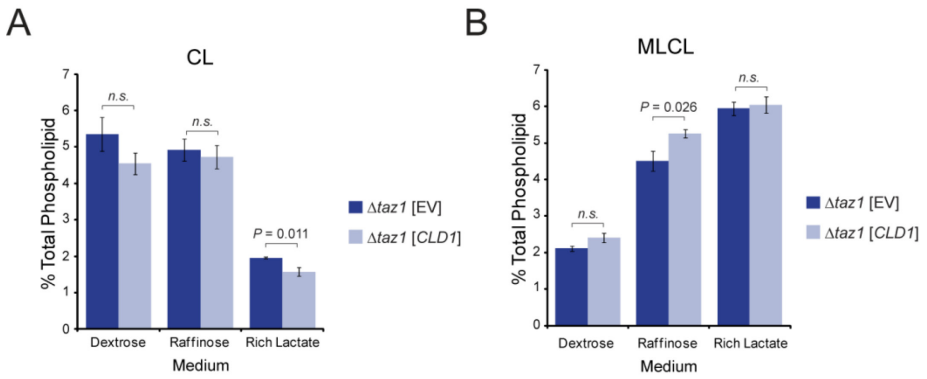

Figure 2.10. CLD1 overexpression. (A) CL or (B) MLCL labeled with $^{32}P_i$ from Δtaz1 yeast transformed with an empty vector (EV) or CLD1 grown in the indicated media was separated by TLC and quantified (mean ± SEM, n=6). Statistical significance was determined by t-test. n.s. = differences not significant.

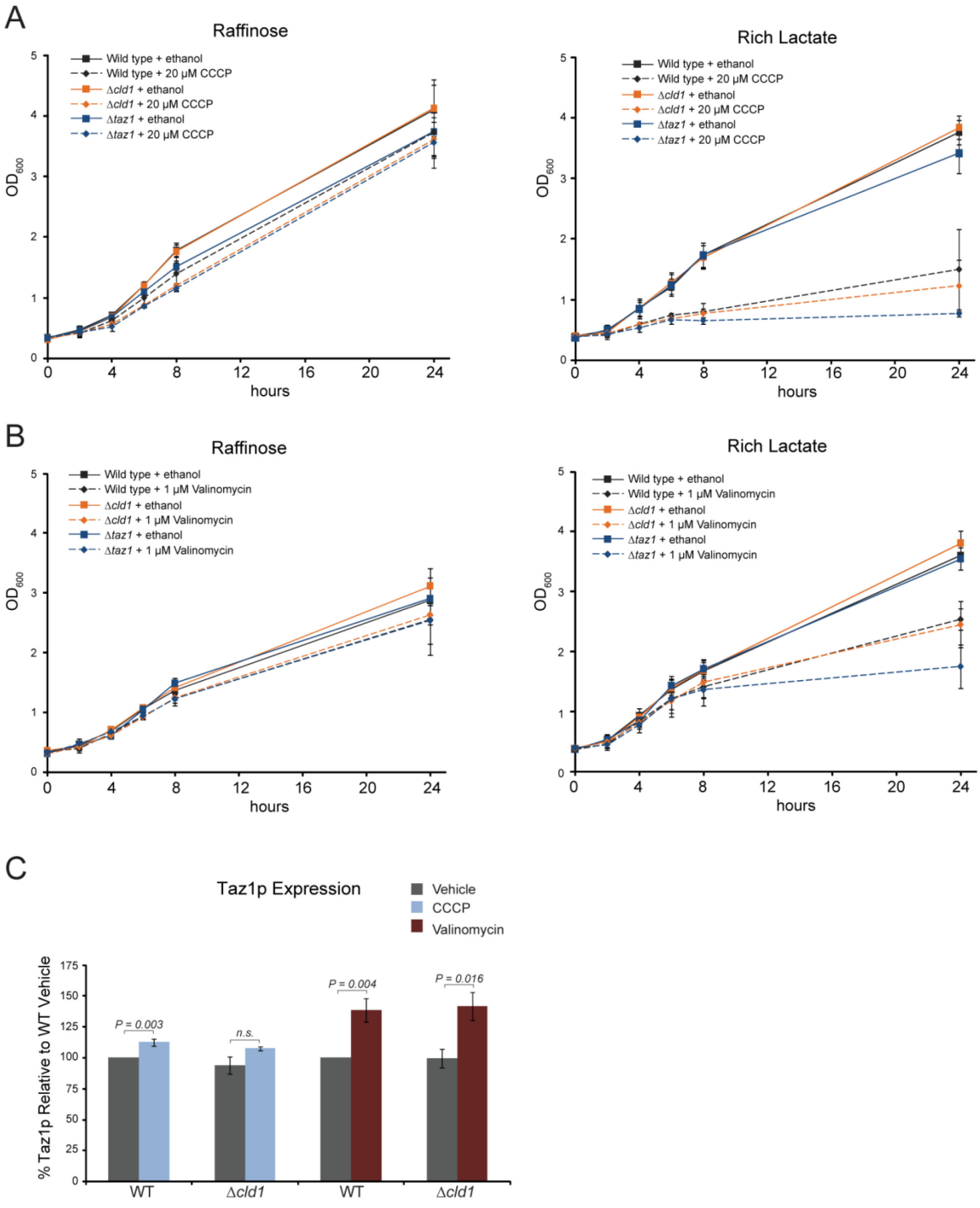
A
Raffinose
Wild type + ethanol
Wild type + 20 μM CCCP
Δcld1 + ethanol
Δcld1 + 20 μM CCCP
Δtaz1 + ethanol
Δtaz1 + 20 μM CCCP
OD600
hours
Rich Lactate
B
Raffinose
Wild type + ethanol
Wild type + 1 μM Valinomycin
Δcld1 + ethanol
Δcld1 + 1 μM Valinomycin
Δtaz1 + ethanol
Δtaz1 + 1 μM Valinomycin
Rich Lactate
C
Taz1p Expression
Vehicle
CCCP
Valinomycin
% Taz1p Relative to WT Vehicle
P = 0.003
n.s.
P = 0.004
P = 0.016
WT
Δcld1
WT
Δcld1

Figure 2.11. Ionophore treatment of yeast. (A and B) Cells were grown in YP-raffinose (left panels) or rich lactate (right panels) for 24 hrs at 30°C, diluted to 0.4 $OD6_{00}$/ml, and treated with (A) 20 μM CCCP or an equal volume of ethanol or (B) 1 μM valinomycin or an equal volume of DMSO. The OD_{600} was measured at the indicated times. Data points represent the mean ± SEM, *n*=4. (C) Taz1p band intensities were quantified and plotted as the % protein relative to wild type grown in the presence of the vehicle (mean ± SEM, *n*=5). Statistical significance was determined by *t*-test. n.s. = differences not significant.

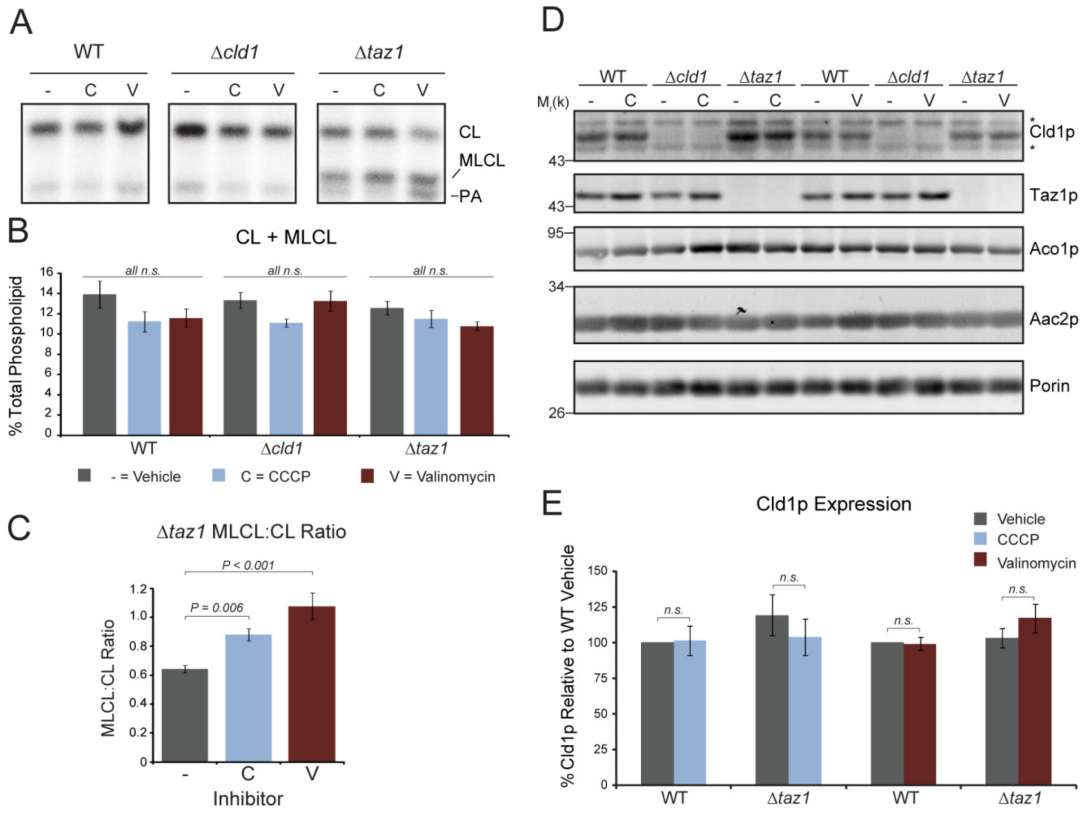

Figure 2.12. Dissipation of the mitochondrial membrane potential promotes Cld1p function. (A) Mitochondrial phospholipids from yeast grown in YP-raffinose spiked with $^{32}P_i$ in the presence of 20 µM CCCP (C), 1 µM valinomycin (V), or vehicle only (-) for 24 hrs were separated by TLC. (B) The sum of CL + MLCL from the indicated strains (mean ± SEM, *n*=6). n.s. = differences not significant as determined by one-way ANOVA. (C) The ratio of MLCL:CL from Δ*taz1* (mean ± SEM, *n*=6). Significant differences determined by one-way ANOVA. (D) Whole cell extracts from the indicated strains grown in the presence of CCCP, valinomycin, or vehicle alone for 24 hrs were resolved by SDS-PAGE and immunoblotted as indicated. *indicates non-specific cross reactions of the Cld1p antiserum. (E) Cld1p band intensities were quantified and plotted as the % Cld1p relative to wild type grown in the absence of either ionophore (mean ± SEM, *n*=5). n.s. = differences not significant as determined by *t*-test.

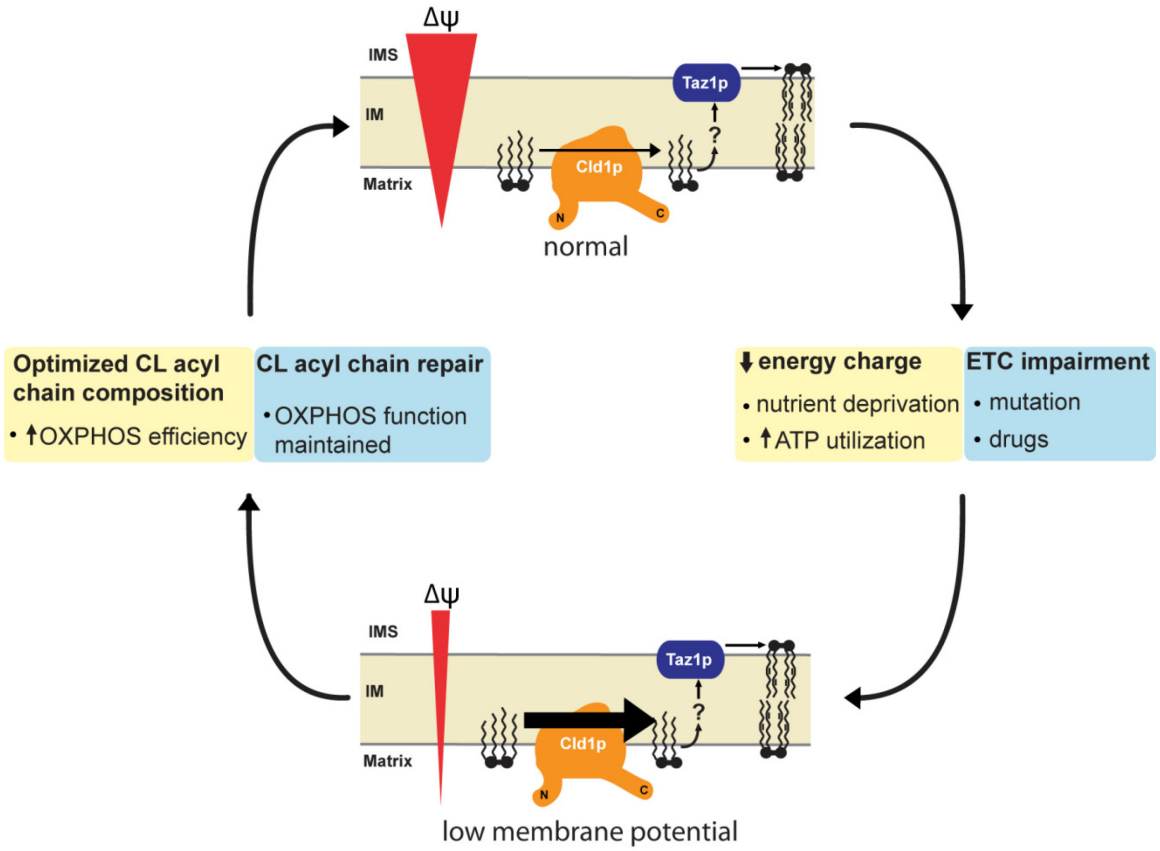
Δψ
IMS
IM
Matrix
Taz1p
Cld1p
N
C
?
normal
Optimized CL acyl chain composition
• ↑OXPHOS efficiency
CL acyl chain repair
• OXPHOS function maintained
↓energy charge
• nutrient deprivation
• ↑ATP utilization
ETC impairment
• mutation
• drugs
Δψ
IMS
IM
Matrix
Taz1p
Cld1p
N
C
?
low membrane potential

Figure 2.13. A feedback loop between OXPHOS and CL remodeling. CL remodeling is enhanced upon dissipation of the mitochondrial membrane potential ($\Delta\psi$). Two potential underlying causes for a drop in the mitochondrial membrane potential include: 1) A decreased energy charge (yellow boxes), or 2) Impairment of the electron transport chain (ETC), either through mutation or pharmacological insult (blue boxes). A reduced mitochondrial membrane potential will increase the rate of CL remodeling. The resultant additional mature CL may increase the efficiency of OXPHOS and thus re-establish the $\Delta\psi$ (yellow boxes). Alternatively, the increased production of ROS that occurs when proton pumping by the electron transport chain is reduced/impaired may oxidize CL. The increased rate of CL remodeling stimulated by the associated reduction in $\Delta\psi$ may therefore replace oxidized acyl chains in CL with new acyl chains, thus preserving OXPHOS function (blue boxes).

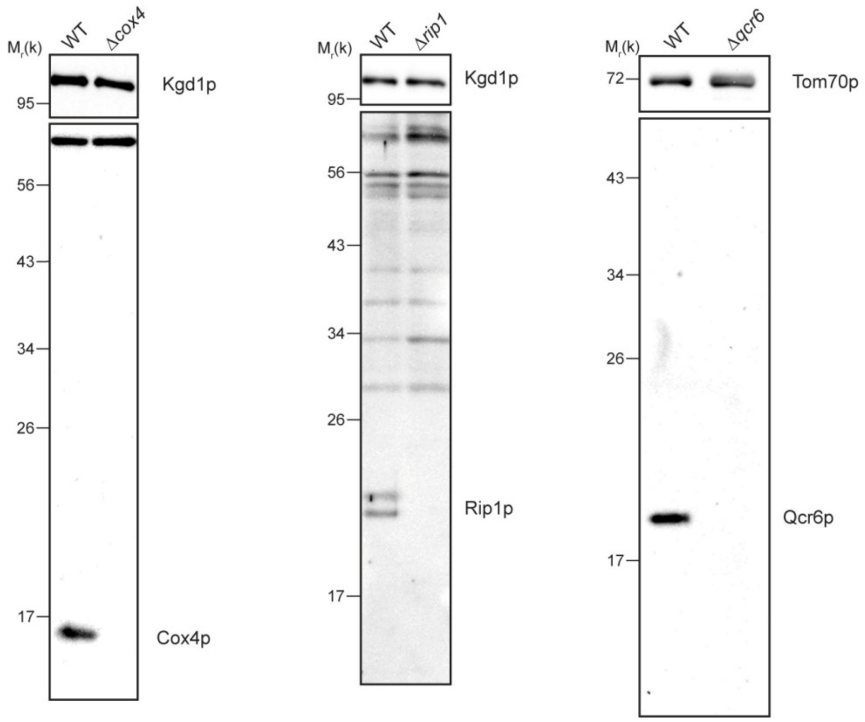

Figure 2.14. Antibody specificity. 25 μg of isolated mitochondria from the indicated strain were immunoblotted using antisera raised against Cox4p, Rip1p, or Qcr6p. Kgd1p or Tom70p served as a loading control

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Chapter 3

Unremodeled and remodeled cardiolipin are functionally indistinguishable in yeast

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SUMMARY

After biosynthesis, an evolutionarily conserved acyl chain remodeling process generates a final highly homogenous and yet tissue-specific molecular form of the mitochondrial lipid cardiolipin. Hence cardiolipin molecules in different organisms, and even different tissues within the same organism, contain a distinct collection of attached acyl chains. This observation is the basis for the widely accepted paradigm that the acyl chain composition of cardiolipin is matched to the unique mitochondrial demands of a tissue. For this hypothesis to be correct, cardiolipin molecules with different acyl chain compositions should have distinct functional capacities and cardiolipin that has been remodeled should promote cardiolipin-dependent mitochondrial processes better than its unremodeled form. However, functional disparities between different molecular forms of cardiolipin have never been established. Here we interrogate this simple but crucial prediction utilizing the best available model to do so, *Saccharomyces cerevisiae*. Specifically, we compare the ability of unremodeled and remodeled cardiolipin, which differ markedly in their acyl chain composition, to support mitochondrial activities known to require cardiolipin. Surprisingly, defined changes in the acyl chain composition of cardiolipin do not alter either mitochondrial morphology or oxidative phosphorylation. Importantly, preventing cardiolipin remodeling initiation in yeast lacking *TAZ1*, an ortholog of the causative gene in Barth syndrome, ameliorates mitochondrial dysfunction. Thus, our data do not support the prevailing hypothesis that unremodeled cardiolipin is functionally distinct from remodeled cardiolipin, at least for the functions examined, suggesting alternative physiological roles for this conserved pathway.

INTRODUCTION

Cardiolipin (CL), a mitochondrial phospholipid with two phosphate headgroups and four acyl chains, is required for the optimal function of numerous mitochondrial processes, including oxidative phosphorylation (OXPHOS) (Acehan et al., 2011; Claypool et al., 2008b; Eble et al., 1990; Fry and Green, 1980; Fry and Green, 1981; Gomez and Robinson, 1999; Koshkin and Greenberg, 2000; Koshkin and Greenberg, 2002; Pfeiffer et al., 2003; Schwall et al., 2012; Sedlak and Robinson, 1999; Zhang et al., 2002; Zhang et al., 2005), protein import (Gebert et al., 2009; Jiang et al., 2000; Marom et al., 2009; van der Laan et al., 2007), establishment of cristae morphology (Claypool et al., 2006; Mileykovskaya and Dowhan, 2009), fission and fusion (Ban et al., 2010; DeVay et al., 2009; Montessuit et al., 2010), and apoptosis (Gonzalvez et al., 2008; Ostrander et al., 2001). Despite limited acyl chain specificity of the CL biosynthetic enzymes (de Kroon et al., 2013; Houtkooper et al., 2006), the acyl chain composition of CL within an organism or cell type displays a remarkable degree of homogeneity (Schlame et al., 2005). This is achieved via acyl chain remodeling that is initiated by a lipase(s), generating monolyso-CL (MLCL, CL lacking one acyl chain), and completed by a transacylase or an acyltransferase which reacylates MLCL (Claypool and Koehler, 2012).

Mutations in the MLCL transacylase tafazzin cause Barth syndrome, resulting in cardiac and skeletal myopathy, cyclic neutropenia, and respiratory chain defects (Barth et al., 1983; Bione et al., 1996; Schlame and Ren, 2006). In Barth syndrome patients and models of Barth syndrome, CL levels are decreased, MLCL accumulates, and the remaining CL contains an altered acyl chain composition (Gu et al., 2004; Schlame et al., 2003; Soustek et al., 2011; Valianpour et al., 2005; Whited et al., 2012; Xu et al., 2006a),

although which lipid alteration either individually or in combination leads to mitochondrial dysfunction has not been thoroughly investigated.

While the pathophysiological importance of defective CL remodeling is firmly established, the physiological importance of this pathway represents a largely unaddressed issue that is preventing a comprehensive molecular understanding of how this evolutionarily conserved and clinically relevant process promotes mitochondrial function. The most widely accepted hypothesis is based on the highly intriguing observation that the final homogeneous molecular form of CL varies between organisms or even between tissues within the same organism (Cheng et al., 2008; Schlame et al., 2005). As such, it is postulated that CL molecules with different acyl chain compositions are functionally distinct, and that the molecular form of CL specifically fits the demands of its host cell (Cheng et al., 2008; Houtkooper et al., 2009; Kiebish et al., 2012; Schlame et al., 2002). However, as of yet, this provocative hypothesis has not been directly tested in any model organism.

At present, yeast are not merely the best, but also the only model available capable of directly comparing the functionality of distinct molecular forms of CL in otherwise isogenic cells. Three CL remodeling pathways have been identified in higher eukaryotes, although their relative contribution to establishing the final molecular form of CL is unclear (Claypool and Koehler, 2012; Schlame, 2013). In contrast, yeast only undergo tafazzin-mediated CL remodeling. Recently, the phospholipase that initiates CL remodeling was identified in yeast as Cld1p (Beranek et al., 2009). Cld1p has no homolog in higher eukaryotes; however its function, the removal of an acyl chain from CL to form MLCL, is conserved. A similar phenomenon is seen with the

phosphatidylglycerolphosphate phosphatase in CL biosynthesis: the yeast (Gep4p) and mammalian (PTPMT1) enzymes are phylogenetically unrelated despite catalyzing the same reaction (Osman et al., 2010; Zhang et al., 2011a). A calcium-independent phospholipase A_2 has been identified as a CL phospholipase in flies ($iPLA_2$-VIA) and mammals ($iPLA_2\gamma$) (Malhotra et al., 2009; Mancuso et al., 2007; Schlame et al., 2012b). However, murine $iPLA_2\gamma$ is not the lipase that provides the substrate MLCL utilized by tafazzin (Kiebish et al., 2013). Consequently, the exact role of these lipases in CL remodeling is unclear. Therefore, in higher eukaryotes, not only are there multiple potential CL remodeling pathways, but additionally, a complete inventory of all of the involved players has not been established. As such, it is currently not possible to compare the functionality of distinct molecular forms of CL in metazoans.

In contrast, Cld1p localizes exclusively to mitochondria and is the only lipase that initiates tafazzin-mediated CL remodeling in yeast (Baile et al., 2013; Beranek et al., 2009). Here, we utilized Δ*cld1* yeast to determine if cardiolipin molecules with different acyl chain compositions—in this case unremodeled *versus* remodeled cardiolipin—have distinct functional capacities, a central prediction of the prevailing hypothesis. Unexpectedly, unremodeled CL functioned as well as remodeled CL in maintaining mitochondrial morphology and promoting OXPHOS. Further, mutating *CLD1*, and thus preventing the initiation of CL remodeling, was able to suppress the defects of Δ*taz1* yeast. Thus, we conclude that in yeast, unremodeled CL can support known CL-dependent mitochondrial functions as well as remodeled CL.

RESULTS

CLD1 functions upstream of TAZ1 in CL remodeling

The initial characterization of *CLD1* revealed that Δ*cld1* and Δ*cld1*Δ*taz1* yeast contained identical mitochondrial phospholipid profiles (Beranek et al., 2009), indicating that *CLD1* is epistatic to *TAZ1* (the yeast homolog of tafazzin) in the same pathway. In contrast, growth on respiratory media, where ethanol and glycerol are the only available carbon sources thus requiring ATP generated by OXPHOS, suggested that *CLD1* functions in a pathway parallel to, or distinct from, *TAZ1* (Beranek et al., 2009).

In an attempt to resolve this, we analyzed CL biosynthesis and remodeling mutants in multiple independent yeast strains. As expected, in mitochondria isolated from yeast lacking cardiolipin synthase (Δ*crd1*), CL was absent and its precursor PG accumulated (Figure 3.1). Importantly, the same phospholipid profile was seen in the double mutant, Δ*crd1*Δ*cld1*, indicating that *CLD1* functions after *CRD1,* in one pathway. The Δ*cld1* mutant displayed a lipid profile similar to that of wild type, while Δ*taz1* resulted in a reduction of CL and an accumulation of MLCL. The double mutant Δ*cld1*Δ*taz1* phenocopied Δ*cld1* and wild type. Thus, in agreement with previous studies (Baile et al., 2013; Beranek et al., 2009), analysis of mitochondrial phospholipids indicates that *CLD1* functions upstream of *TAZ1* and downstream of *CRD1* in the CL remodeling pathway.

The placement of *CLD1* between *CRD1* and *TAZ1* was also analyzed by growth on respiratory media (Figure 3.2). Δ*crd1* yeast displayed a growth defect, and both Δ*crd1*Δ*cld1* and Δ*crd1*Δ*taz1* mimicked this phenotype, confirming that *CRD1* is upstream of both *CLD1* and *TAZ1*. Δ*taz1* yeast also displayed a growth defect on

respiratory media, but only at elevated temperature. Consistent with Berenak *et al*, Δ*cld1* yeast grew as well as wild type, but in contrast to their results, our analysis showed that Δ*cld1*Δ*taz1* did not exhibit a growth defect and phenocopied Δ*cld1*. To rule out strain-specific differences, this epistasis analysis was confirmed in two additional genetic backgrounds (Figures 3.1 and 3.2). Therefore, *CLD1* functions upstream of *TAZ1* in a single biochemical pathway.

Δcld1 mitochondria contain unremodeled CL

CL from Δ*cld1* yeast was previously shown by GC/MS to contain more $C_{16:0}$ fatty acyl chains than wild type at the expense of $C_{18:1}$ and $C_{16:1}$ (Beranek et al., 2009). This is consistent with the presence of unremodeled CL in Δ*cld1* mitochondria, and prompted us to analyze more comprehensively the acyl chain composition of CL in remodeling mutants by shotgun lipidomics (Figure 3.3A and Tables 3.1 and 3.2). The acyl chain composition of CL from Δ*cld1* or Δ*taz1* mitochondria was clearly altered compared to wild type, and the CL species that accumulated in Δ*cld1* were similar to that of Δ*cld1*Δ*taz1*, consistent with *CLD1* functioning upstream of *TAZ1*.

Unremodeled CL in yeast is characterized in part by saturated acyl chains of increased heterogeneity. To quantify this, the molecular species of CL from each strain was categorized by the number of saturated acyl chains, and expressed as the percent of total CL (Figure 3.3B). CL from wild type mitochondria contained mostly unsaturated fatty acyl chains; only 8% contained one saturated acyl chain while the remaining 92% of CL contained no saturated acyl chains. In contrast, only 20% of CL from Δ*cld1* contained no unsaturated acyl chains, whereas 51% and 28% of CL contained one or two saturated acyl chains, respectively.

Interestingly, the presence of CL with no saturated acyl chains in both Δ*cld1* and Δ*taz1* mitochondria suggests that either an alternate (albeit minor) CL remodeling pathway exists, or instead that a subpopulation of newly synthesized CL already contains four unsaturated acyl chains. While the relative amounts of mature (e.g. a remodeled-like acyl chain composition) CL vary between Δ*cld1* and Δ*taz1* mitochondria, the absolute amounts are similar (Figure 3.3C and D), implying that Cld1p is able to specifically deacylate unremodeled CL. This is further supported by the molecular species of MLCL present in Δ*taz1*; although 28% of CL in Δ*cld1* contains 2 saturated acyl chains, none of the molecular forms of MLCL in Δ*taz1* contained 2 saturated acyl chains (Figure 3.3E and Table 3.2), suggesting that Cld1p preferentially removes saturated acyl chains from CL.

Thus, we have provided the most extensive analysis to date of the CL acyl chain composition in CL remodeling mutants. CL molecules from Δ*cld1* yeast contain more saturated acyl chains than wild type, consistent with unremodeled CL. Accordingly, Δ*cld1* is a genetic tool to determine if CL molecules with different acyl chain compositions are functionally distinct in a strain that is otherwise isogenic to wild type yeast.

CL remodeling is not required to maintain mitochondrial morphology

Altered mitochondrial morphology has been observed in Δ*crd1* and Δ*taz1* yeast (Claypool et al., 2008a; Mileykovskaya and Dowhan, 2009). To determine what role, if any, CL remodeling plays in the establishment and/or maintenance of mitochondrial morphology, CL remodeling mutants were analyzed by EM (Figures 3.4 and 3.5).

No overt morphological differences were observed between mitochondria in wild type and Δ*cld1* yeast (Figure 3.4A). Surprisingly, the morphology of mitochondria in Δ*crd1* and Δ*taz1* also appeared unaffected, although measurement of mitochondrial membranes indicated that both mutants contained longer cristae membranes than wild type or Δ*cld1* (Figure 3.4B and C). Additionally, no difference in the number of aberrant mitochondria, which display exaggerated cristae, was observed between wild type and any CL remodeling mutant (Figure 3.4D and E).

To confirm these results, mitochondria from CL remodeling mutants derived from the W303 genetic background were also analyzed (Figure 3.5). While no remarkable morphological defects were observed (Figure 3.5A), mitochondria from Δ*crd1* and Δ*cld1* (but not Δ*taz1*) yeast displayed longer cristae membranes than wild type, and all of the mutants displayed longer OM membranes than wild type (Figure 3.5B and C). Additionally, in the W303 background, no aberrant mitochondria were observed in wild type (Figure 3.5D and E), unlike the mutants which contained a small fraction of aberrant mitochondria.

The mitochondrial morphology in Δ*crd1,* Δ*cld1*, and Δ*taz1* yeast remained largely unperturbed, although subtle differences were noted. Importantly, studies which previously reported abnormal mitochondrial morphology in Δ*crd1* and Δ*taz1* yeast never reported the penetrance of the observed defects (Claypool et al., 2008a; Mileykovskaya and Dowhan, 2009). Furthermore, our results indicate that the genetic background contributes to mitochondrial morphology. Thus, we conclude that there is not a general morphological phenotype in mitochondria lacking either remodeled CL or CL entirely.

CL remodeling is not required for optimal OXPHOS function

CL is required for the optimal function of respiratory complexes (Fry and Green, 1980; Fry and Green, 1981; Gomez and Robinson, 1999; Koshkin and Greenberg, 2000; Koshkin and Greenberg, 2002; Schwall et al., 2012; Sedlak and Robinson, 1999), as well as for the stability of respiratory supercomplexes (Claypool and Koehler, 2012; Claypool et al., 2008b; Pfeiffer et al., 2003; Zhang et al., 2002; Zhang et al., 2005). Respiratory supercomplexes, which in yeast consist of two copies of complex III and either one (III_2IV) or two (III_2IV_2) copies of complex IV, increase the efficiency of electron flux through the electron transport chain *via* substrate channeling (Cruciat et al., 2000; Lapuente-Brun et al., 2013). Thus, we used Δ*cld1* to determine the ability of unremodeled CL to support OXPHOS.

In Δ*crd1* (and Δ*crd1*Δ*cld1*) mitochondria, respiratory supercomplexes were destabilized due to the absence of CL, as seen by the decreased abundance of the III_2IV_2 supercomplex and the resultant increase in the III_2IV supercomplex, as well as the liberated complex III dimer and free complex IV (Figure 3.6A). Additionally, the ADP/ATP carrier (Aac2p) did not assemble into higher molecular weight complexes, including with respiratory complexes, in the absence of CL (Claypool et al., 2008b). In Δ*taz1*, respiratory supercomplex stability was not affected, although the association of Aac2p with the supercomplex was diminished. In Δ*cld1* however, the stability of respiratory supercomplexes, including those containing Aac2p, was preserved, indicating that the acyl chain composition of CL does not affect respiratory supercomplex stability.

We further investigated the role of the molecular form of CL in OXPHOS by measuring the rate of O_2 consumption in isolated mitochondria. The P/O ratio (a measure of OXPHOS efficiency) in Δ*cld1* mitochondria was indistinguishable from wild type, but

was decreased in Δ*crd1*, Δ*crd1*Δ*cld1*, and Δ*taz1* mitochondria (Figure 3.6B). Likewise, no change in the respiratory control ratio (RCR; a measure of OXPHOS coupling) was observed in Δ*cld1* compared to wild type (Figure 3.6C). Notably, the OXPHOS defects observed in Δ*taz1* mitochondria were suppressed after the additional deletion of *CLD1*.

As an alternative method to measure OXPHOS function, we tracked the membrane potential ($\Delta\psi_m$) of isolated mitochondria using the potentiometric fluorescent probe TMRM (Figure 3.6D). $\Delta\psi_m$ was established *via* NADH addition, and state 3 respiration was induced by adding ADP, which caused a transient depolarization due to the utilization of the proton gradient to drive ADP/ATP transport by Aac2p and ATP production by complex V. After the ADP was consumed, the inner membrane repolarized and state 4 respiration resumed. Δ*cld1* and Δ*cld1*Δ*taz1* mitochondria were able to repolarize at rates identical to wild type, whereas Δ*crd1*, Δ*crd1*Δ*cld1* and Δ*taz1* mitochondria repolarized more slowly (Figure 3.6E). Taken together, these results indicate that OXPHOS coupling is not dependent on the acyl chain composition of CL that is generated by tafazzin-mediated remodeling.

Interestingly, the individual state 3 and state 4 respiration rates in Δ*crd1* and Δ*taz1* mitochondria were higher than in wild type (Figure 3.7A), which is consistent with some reports, but different from others (Claypool et al., 2008b; Jiang et al., 2000; Koshkin and Greenberg, 2000; Koshkin and Greenberg, 2002; Ma et al., 2004). This observation potentially could be due to a lower steady-state $\Delta\psi_m$ for mitochondria from these strains which, due to respiratory control, would result in higher respiration rates (e.g. easier to pump protons against a lower electrochemical potential); but relative $\Delta\psi_m$ measurements in Δ*crd1* and Δ*crd1*Δ*cld1* mitochondria were not significantly different

from wild type (Figure 3.7B). However, TMRM time traces revealed that immediately after establishing the $\Delta\psi_m$, Δ*crd1*, Δ*crd1*Δ*cld1,* and Δ*taz1* mitochondria began to depolarize, whereas those from the other strains maintained a high $\Delta\psi_m$ (Figure 3.6D). This depolarization may originate from breaches in the inner membrane permeability barrier that, although not large enough to resolve on measurements of relative steady-state $\Delta\psi_m$ (Figure 3.7B), could be resolved on individual traces (Figure 3.6D). To identify the source of the putative proton leak, we tested the effects of inhibiting two key OXPHOS components, complex V using oligomycin and Aac2p using carboxyatracyloside (Figure 3.7C); both of which create regulated aqueous conduits in the membrane and require CL for assembly (Acehan et al., 2011; Claypool et al., 2008b). The lack of transient depolarization after ADP addition confirmed the efficacy of both oligomycin and carboxyatracyloside. Interestingly, both inhibitors curtailed the immediate depolarization in Δ*crd1*, Δ*crd1*Δ*cld1* and Δ*taz1* mitochondria, suggesting that in these strains, the time-dependent decrease in $\Delta\psi_m$ was mediated by proton leak through complex V and Aac2p.

When respiration was analyzed under uncoupled conditions to measure maximum electron transport capacity, a small but significant decrease in mitochondria lacking *CRD1* was measured, but no decrease was seen in Δ*taz1* or Δ*cld1* (Figure 3.8A). Measurement of individual complex III and complex IV activities revealed that the defect was specific to complex III (Figure 3.8B and C), consistent with CL participating in its catalytic mechanism (Wenz et al., 2009). Further, the steady state abundance of respiratory complex subunits (as well as other mitochondrial proteins) was not affected in any mutant (Figure 3.8D). Thus, unremodeled and remodeled CL, which differ

significantly in their acyl chain composition, have the same capacity to promote the expression, assembly, and activity of the OXPHOS system.

DISCUSSION

Despite the pervasiveness of the hypothesis that CL remodeling establishes a molecular form of CL that is optimized to support mitochondrial function, direct evidence for this proposition is lacking. Using Δ*cld1* yeast, which cannot initiate CL remodeling, we have provided the most comprehensive comparison to date of the intrinsic functional capacity of distinct molecular forms of CL—remodeled *vs*. unremodeled CL—in otherwise isogenic cells. Our data indicate that in yeast unremodeled and remodeled CL are equally able to maintain mitochondrial morphology and promote OXPHOS and are thus at variance with the prevailing model that CL remodeling is critical for mitochondrial function. Still, it is possible that the acyl chain composition in mammals plays a larger role in controlling OXPHOS function than in yeast and that this capacity has been a relatively recent addition to the functionality of this remodeling pathway. Consistent with this possibility is that cardiolipin remodeling attributed to acyl-CoA:lysocardiolipin acyltransferase-1, which localizes to the endoplasmic reticulum, is associated with mitochondrial dysfunction (Cao et al., 2004; Cao et al., 2009; Li et al., 2010). Alternatively, mitochondrial processes other than OXPHOS that are presently not known and thus not interrogated in the present study, may be dependent on a specific CL acyl chain composition.

Our results, however, suggest that CL remodeling evolved to achieve other biological outcomes instead of simply establishing a tissue-specific molecular form of CL. The ability to remodel CL acyl chains may be more important than the establishment of a specific molecular form. CL is susceptible to oxidative damage due to its tight association with respiratory complexes, the major sites of reactive oxygen species

production in a cell (Kim et al., 2011). Thus, CL remodeling may be used as a repair mechanism that removes and replaces damaged acyl chains, restoring OXPHOS capacity (Baile et al., 2013; Musatov, 2006). Indeed, increased oxidative damage is observed in Δ*taz1* yeast and Barth syndrome lymphoblasts (Chen et al., 2008; Gonzalvez et al., 2013). Why then, do most tissues/organisms contain only a few molecular forms of CL? Tafazzin has no acyl chain specificity (Xu et al., 2006b). Thus, the acyl chain composition of remodeled CL may instead reflect the acyl chain composition of the surrounding lipids in the microenvironment containing tafazzin (Schlame et al., 2012a). Additionally, when compared to Δ*cld1*, the acyl chain composition of CL in Δ*taz1* suggests that saturated acyl chains are the preferred substrate of Cld1p. As such, the specificity of the lipase may also contribute to the final molecular form of CL in a given tissue/cell (Zhang et al., 2011b).

These results have important implications regarding the pathological causes of Barth syndrome. Great emphasis has been placed on the altered CL acyl chain composition, but Barth syndrome patients (and models) also exhibit decreased levels of CL with concurrent increases in MLCL (Gu et al., 2004; Schlame et al., 2003; Soustek et al., 2011; Valianpour et al., 2005; Whited et al., 2012; Xu et al., 2006a). Our data suggest that the absolute levels of lipids (either decreased CL or increased MLCL) and/or the absence of an active remodeling pathway may exert a larger role in contributing to the disease state than simple changes in the final acyl chain composition. These conclusions have therapeutic implications. For instance, if alterations in the levels of CL and MLCL are the major drivers of mitochondrial dysfunction, than therapies promoting the accumulation of CL and/or depletion of MLCL may alleviate the symptoms of Barth

syndrome. An obvious target to inhibit is the lipase that initiates CL remodeling, as we have shown that the Δ*cld1*Δ*taz1* yeast strain phenocopies wild type. Interestingly, Barth syndrome patient lymphoblasts treated with the $iPLA_2$ inhibitor bromoenol lactone partially corrects the MLCL:CL ratio, as does knocking out $iPLA_2$ in $TAZ^{-/-}$ flies (Malhotra et al., 2009). However, this strategy is currently hampered in patients since the relevant lipase(s) that functions upstream of tafazzin has not been molecularly identified (Kiebish et al., 2013). Thus further investigation into the basic biology of CL remodeling is required before plausible treatments can be realized.

MATERIALS AND METHODS

Yeast strains and growth conditions

All yeast strains used in this study were isogenic to GA74-1A (*MAT***a**, *his3-11,15*, *leu2*, *ura3*, *trp1*, *ade8* [rho^+, mit^+]) except where indicated, and have been described previously (Baile et al., 2013; Claypool et al., 2008a; Claypool et al., 2008b; Jarosch et al., 1996), except Δ*crd1*Δ*cld1*, which was generated by replacing the entire open reading frame of *CLD1* with *HIS3MX6* and *CRD1* with *TRP1* (Wach et al., 1994). Strains derived from W303 (*MAT***a**, *ade2-1, ura3-1,15, his3-11, trp1-1, can1-100* [rho^+, mit^+]) were generated by replacing *CRD1* with *TRP1*, *CLD1* with *HIS3MX6*, and/or *TAZ1* with *URA3MX*. Strains derived from PTY144 (*MAT*α, *leu2-3,112, ura3-52, trp1-*Δ*1, lys2, his3::hisg* [rho^+, *LYS2*]) (Thorsness et al., 2002) were generated by replacing *CRD1*, *CLD1*, or *TAZ1* with *HIS3MX6*, except Δ*cld1*Δ*taz1* in which *CLD1* was replaced with *HIS3MX6* and *TAZ1* with *URA3MX*. Yeast were grown in rich lactate media (1% yeast extract, 2% tryptone, 0.05% dextrose, 2% lactic acid, 3.4 mM $CaCl_2$-$2H_2O$, 8.5 mM NaCl, 2.95 mM $MgCl_2$-$6H_2O$, 7.35 mM KH_2PO_4, 18.7 mM NH_4Cl, pH 5.5), except for growth analysis where overnight YPD (1% yeast extract, 2% peptone, 2% dextrose) cultures were spotted on synthetic media (0.17% yeast nitrogen base, 0.5% ammonium sulfate, 0.2% complete amino acid mix with either 2% dextrose or 3% glycerol/1% ethanol). Genetic knockouts constructed for this study were generated by replacing the entire open reading frame of the gene using PCR-mediated gene replacement (Wach et al., 1994).

Multidimensional mass spectrometry-based shotgun lipidomic analysis of mitochondrial lipids

A modified Bligh and Dyer procedure was used to extract lipids from each yeast mitochondrial preparation. Each lipid extract was reconstituted with a volume of 200 µl/mg mitochondrial protein in chloroform/methanol (1:1; v/v). Internal standards for quantification of individual molecular species of lipid classes were added prior to lipid extraction (Christie and Han, 2010). Shotgun lipidomics analyses were performed with a QqQ mass spectrometer (Thermo Fisher Scientific TSQ Vantage, San Jose, CA) equipped with an automated nanospray device (Triversa Nanomate, Advion Biosciences, Ithaca, NY) and operated with Xcalibur software as previously described (Han et al., 2008). Identification and quantification of lipid molecular species were performed using an automated software program as previously described (Yang et al., 2009).

Electron Microscopy

Cells were harvested and fixed in 3% glutaraldehyde contained in 0.1 M Na cacodylate, pH 7.4, 5 mM $CaCl_2$, 5 mM $MgCl_2$, and 2.5% (w/v) sucrose for 1 hour at room temperature with gentle agitation; spheroplasted; embedded in 2% ultra low temperature agarose (prepared in water); cooled; and subsequently cut into small pieces (~$1mm^3$). The cells were then post-fixed in 1% OsO_4, 1% potassium ferrocyanide contained in 0.1 M Na cacodylate, 5mM $CaCl_2$, pH 7.4 for 30 minutes at room temperature. The blocks were washed thoroughly 4X with ddH_20, 10 minutes total; transfered to 1% thiocarbohydrazide at room temperature for 3 minutes; washed in ddH_2O (4X, 1 min each); and transferred to 1% OsO_4, 1% potassium ferrocyanide in 0.1 M Na cacodylate, pH 7.4 for an additional 3 min at room temperature. The cells were

washed 4X with ddH_2O (15 minutes total); en bloc stained in Kellenberger's uranyl acetate (UA) for 2 hr to overnight; dehydrated through a graded series of ethanol; and subsequently embedded in Spurr resin. Sections were cut on a Reichert Ultracut T ultramicrotome; post-stained with UA and lead citrate; and observed on an FEI Tecnai 12 TEM at 100kV. Images were recorded with a Soft Imaging System Megaview III digital camera; and figures were assembled in Adobe Photoshop with only linear adjustments in contrast and brightness.

Assessment of $\Delta\psi_m$

The lipophilic cationic dye tetramethylrhodamine methyl ester (TMRM, Molecular Probes), which accumulates in mitochondria in accordance with a Nernstian distribution, was used in quench mode. 2 ml samples of mitochondria (0.1 mg mitochondrial protein per ml) in measurement buffer (MB; 20 mM Tris-HCl, pH 7.2, 20 mM KCl, 3mM $MgCl_2$, 4mM KH_2PO_4 and 250 mM sucrose) containing 50 nM TMRM (from DMSO stocks, final DMSO concentration 1.0% (v/v)) were added to stirred cuvettes. TMRM emission (λ_{ex} 547 nm; λ_{em} 570 nm; slits at 4 nm) was measured over a time course that included the successive addition of: (i) respiratory substrate (2 mM NADH) at 100 s; (ii) 45 µM ADP, pH 7.5 at 300 s and 700 s, and (iii) 2.5 µM valinomycin at 1000 s to completely dissipate the potential. The relative measure of $\Delta\psi_m$ was based on the difference in fluorescence intensity (ΔF) prior to respiratory substrate addition and after establishment of the maximal $\Delta\psi_m$. The time dependence of return to state 4 respiration following the initiation of a phosphorylation cycle was calculated graphically (Kaleidagraph) as the time between ADP addition and stable re-establishment of the maximal $\Delta\psi_m$. Carboxyatractyloside and oligomycin, each at final concentration of

10 μM were incubated with mitochondria at 24C for 5 min either separat ely or together. After incubation, TMRM emission was measured over a time course as described above.

Complex III and complex IV activity measurements

Complex III and IV activities were measured as described (Tzagoloff et al., 1975) with a few modifications. To measure complex III activity, 1-25 μg mitochondria solubilized in 0.5% (w/v) *n*-dodecyl β-D-maltoside were added to reaction buffer (50 mM KP_i, 2 mM EDTA, pH 7.4) with 0.008% (w/v) horse heart cytochrome *c* and 1 mM potassium cyanide. The reaction was started by adding 100 μM decylubiquinol, and the reduction of cytochrome *c* followed at 550 nm. Complex IV activity was measured by adding mitochondrial extracts to reaction buffer with 0.008% (w/v) ferro-cytochrome *c* and following cytochrome *c* oxidation at 550 nm.

Antibodies

Most antibodies used in this study were generated in our laboratory or the Schatz (J. Schatz, University of Basel, Basel, Switzerland) or Koehler (C. Koehler, University of California, Los Angeles, Los Angeles, CA) laboratories and have been described previously (Baile et al., 2013; Claypool et al., 2006; Daum et al., 1982; Maccecchini et al., 1979; Ohashi et al., 1982; Poyton and Schatz, 1975; Riezman et al., 1983; Whited et al., 2012). Other antibodies used were mouse anti-Aac2p clone 6H8 (Panneels et al., 2003), and horseradish peroxidase-conjugated (Thermo Fisher Scientific) or fluorescent-conjugated (Pierce) secondary antibodies.

Miscellaneous

Isolation of mitochondria, preparation of yeast cell extracts, blue native-PAGE, mitochondrial respiration, phospholipid analysis, and immunoblotting were performed as

previously described (Claypool et al., 2008a; Claypool et al., 2006; Claypool et al., 2008b). Statistical comparisons were performed by one-way analysis of variance compared to wild type using SigmaPlot 11 software (Systate Software, San Jose, CA). All graphs show the mean ± SEM.

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TABLES

Table 3.1. Molecular species of CL

Molecular species		60:3	60:2	62:3	62:2	64:4	64:3	64:2	66:4	66:3	66:2	68:4	68:3	68:2	70:4	70:3	72:4	72:3		
Acyl chain comosition		14:0-14:1-16:1-16:1	14:0-14:0-16:1-16:1	14:0-16:1-16:1-16:1	14:0-16:1-16:1-16:0	16:1-16:1-16:1-16:1	16:0-16:1-16:1-16:1, 14:0-16:1-16:1-18:1	16:0-16:1-16:1-16:0, 14:0-16:1-16:1-18:0	16:1-16:1-16:1-18:1	16:0-16:1-16:1-18:1, 14:0-16:1-18:1-18:1, 16:1-16:1-16:1-18:0	16:0-16:0-16:1-18:1, 16:0-16:1-18:1-18:0	16:1-16:1-18:1-18:1	16:0-16:1-18:1-18:1	16:0-16:0-18:1-18:1, 16:0-16:1-18:1-18:0	16:1-18:1-18:1-18:1	16:1-18:1-18:1-18:0, 16:0-18:1-18:1-18:1	18:1-18:1-18:1-18:1	18:1-18:1-18:1-18:0		
Mass		644.92	645.93	658.94	659.95	671.95	672.95	673.96	685.96	686.97	687.98	699.98	700.99	701.99	713.99	715.00	728.01	729.02	Total CL	MLCL:CL
	WT prep 1[a]	0.00		0.19		0.96	0.25		6.42	0.48		15.23	1.06		11.30	0.69	2.94	0.22	39.74	0.022
	WT prep 2	0.07		0.35		0.56	0.37		4.67	0.35		12.01	0.79		11.98	0.85	3.98	0.26	36.24	0.024
	WT prep 3	0.11		0.42		0.71	0.39		5.41	0.22		13.57	0.79		14.12	0.90	3.28	0.24	40.17	0.035
	Δtaz1 prep 1								1.51	0.77		4.01	1.93	0.53	1.25	0.69	0.11	0.09	10.92	2.057
	Δtaz1 prep 2								1.09	0.60		4.14	2.29	0.72	1.50	1.14	0.12	0.14	11.75	1.828
	Δtaz1 prep 3								0.77	0.35		3.17	1.64	0.52	1.10	0.76	0.14	0.12	8.57	2.507
	Δcld1 prep 1		0.00		0.00		0.63	0.60	1.33	4.01	2.68	3.40	7.48	4.64	1.59	3.28	0.24	0.30	30.17	0.012
	Δcld1 prep 2		0.03		0.14		0.60	0.59	1.13	3.88	3.09	3.17	8.22	5.86	1.51	3.37	0.21	0.30	32.11	0.008
	Δcld1 prep 3		0.01		0.08		0.47	0.42	0.91	2.86	2.17	2.73	7.09	4.91	1.46	2.99	0.21	0.29	26.60	0.010
	Δtaz1 Δcld1 prep 1		0.00		0.14		0.53	0.52	1.23	4.22	3.74	3.15	8.97	7.04	1.37	3.32	0.14	0.26	34.63	0.050
	Δtaz1 Δcld1 prep 2		0.04		0.09		0.48	0.54	1.01	3.59	3.22	3.29	8.61	6.96	1.16	3.01	0.11	0.23	32.33	0.045
	Δtaz1 Δcld1 prep 3		0.00		0.00		0.32	0.38	1.00	3.62	2.97	3.48	9.02	7.10	1.26	3.14	0.15	0.25	32.71	0.039
Significance[b]	WT vs taz								yes	no		yes	yes		yes	no	yes	yes	yes	yes
	WT vs cld						no		yes	yes		yes	yes		yes	yes	yes	yes	yes	no
	WT vs cldtaz						no		yes	yes		yes	yes		yes	yes	yes	no	yes	no
	taz vs cld								no	yes		no	yes	yes	no	yes	no	yes	yes	yes
	taz vs cldtaz								no	yes		no	yes	yes	no	yes	no	yes	yes	yes
	cld vs cldtaz		no		no		no	no	no	no	no	no	yes	yes	no	no	no	yes	no	no

[a]nmol CL /mg protein

[b]Significance determined by one-way analysis of variance

Table 3.2. Molecular species of MLCL

Molecular species		46:2	48:3	48:2	50:3	50:2	52:3	52:2	54:3	54:2	
Acyl chain comosition		14:0-16:1-16:1	16:1-16:1-16:1	16:1-16:1-16:0	16:1-16:1-18:1	16:1-16:1-18:0, 16:0-16:1-18:1	16:1-18:1-18:1	16:0-18:1-18:1	18:1-18:1-18:1	18:1-18:1-18:0	
Mass		540.83	553.84	554.85	567.86	568.86	581.87	582.88	595.89	596.89	TOTAL MLCL
	WT prep 1[a]		0.03		0.29		0.41	0.02	0.11		0.85
	WT prep 2		0.03		0.26		0.40	0.03	0.16		0.87
	WT prep 3		0.04		0.42		0.70	0.02	0.21		1.40
	Δtaz1 prep 1	0.05	0.51	0.45	4.30	2.49	8.29	4.24	1.37	0.75	22.45
	Δtaz1 prep 2	0.09	0.29	0.32	3.38	2.22	7.88	4.79	1.54	0.97	21.49
	Δtaz1 prep 3	0.09	0.30	0.31	3.44	2.07	8.22	4.57	1.56	0.91	21.47
	Δcld1 prep 1				0.07	0.13	0.07	0.09			0.35
	Δcld1 prep 2				0.02	0.13	0.03	0.07			0.26
	Δcld1 prep 3				0.02	0.14	0.03	0.09			0.27
	Δtaz1 Δcld1 prep 1			0.09	0.20	0.46	0.38	0.54	0.02	0.04	1.72
	Δtaz1 Δcld1 prep 2			0.05	0.19	0.38	0.31	0.47	0.02	0.04	1.46
	Δtaz1 Δcld1 prep 3			0.01	0.16	0.37	0.27	0.41	0.00	0.05	1.27
Significance[b]	WT vs taz		yes		yes		yes	yes	yes		yes
	WT vs cld				no		yes	no			no
	WT vs cldtaz				no		no	yes	yes		no
	taz vs cld				yes	yes	yes	yes			yes
	taz vs cldtaz			yes	yes	yes	yes	yes	yes	yes	yes
	cld vs cldtaz				no	yes	no	yes			yes

[a]nmol MLCL /mg protein

[b]Significance determined by one-way analysis of variance

FIGURES

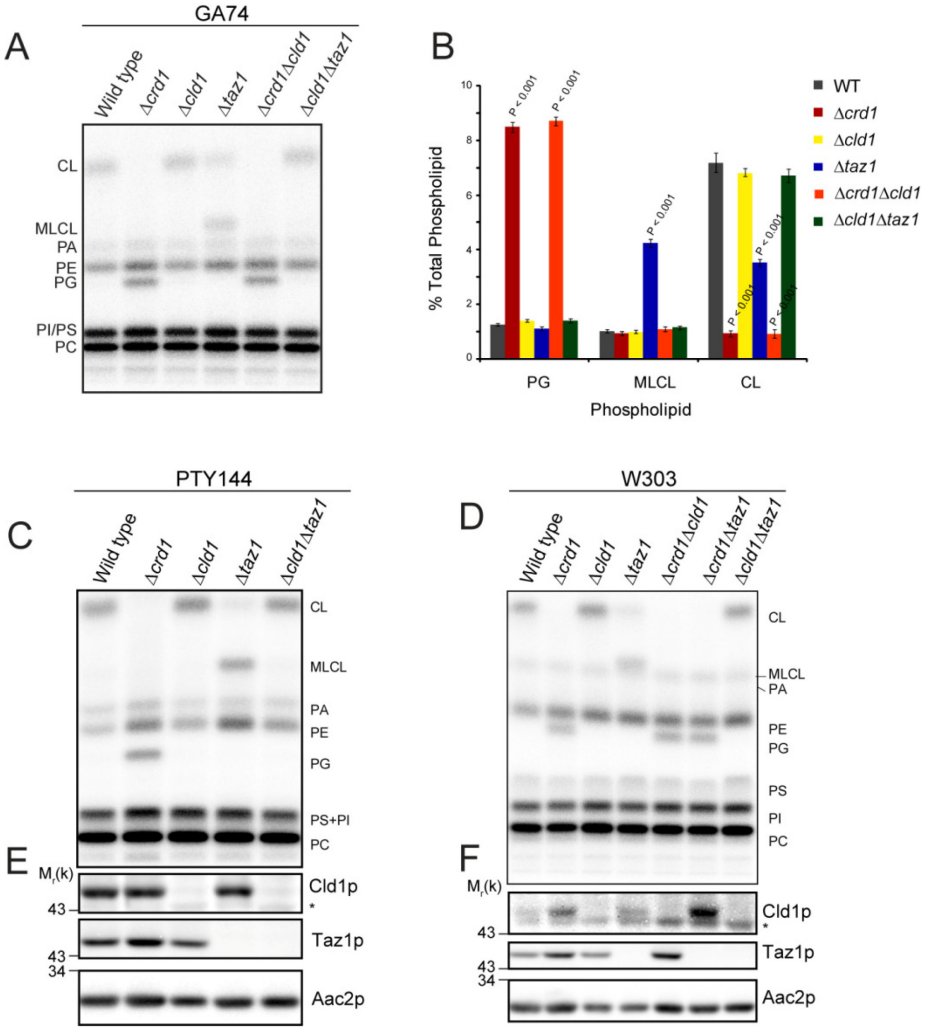

A
GA74
Wild type
Δcrd1
Δcld1
Δtaz1
Δcrd1Δcld1
Δcld1Δtaz1
CL
MLCL
PA
PE
PG
PI/PS
PC
B
% Total Phospholipid
P < 0.001
WT
Δcrd1
Δcld1
Δtaz1
Δcrd1Δcld1
Δcld1Δtaz1
PG
MLCL
CL
Phospholipid
C
PTY144
E
M_r(k)
43
Cld1p
*
Taz1p
34
Aac2p
D
W303
F
PS
PI

Figure 3.1. *CLD1* is epistatic to *TAZ1* in the CL remodeling pathway by mitochondrial phospholipid analysis. (A) Mitochondrial phospholipids from the indicated strains derived from GA74-1A were labeled with $^{32}P_i$ and separated by TLC. (B) Quantification of (A). n = 5-6. *** $p < 0.001$ Mitochondrial phospholipids from the indicated strains derived from (C) PTY144 and (D) W303 were analyzed as in (A). Whole cell extracts from the indicated strains derived from (E) PTY144 and (F) W303 were immunoblotted. * indicates a non-specific cross reaction of the Cld1p antisera. PA, phosphatidic acid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine.

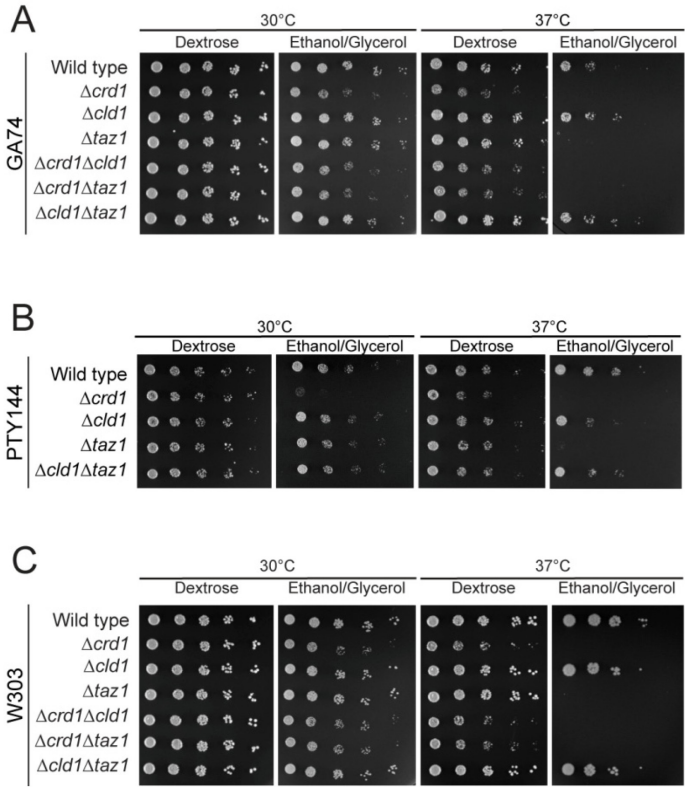


Figure 3.2. *CLD1* is epistatic to *TAZ1* in the CL remodeling pathway by respiratory growth analysis. 1:4 serial dilutions of the indicated strains derived from (A) GA74-1A, (B) PTY144, or (C) W303 were spotted on the indicated media and incubated at 30°C or 37°C.

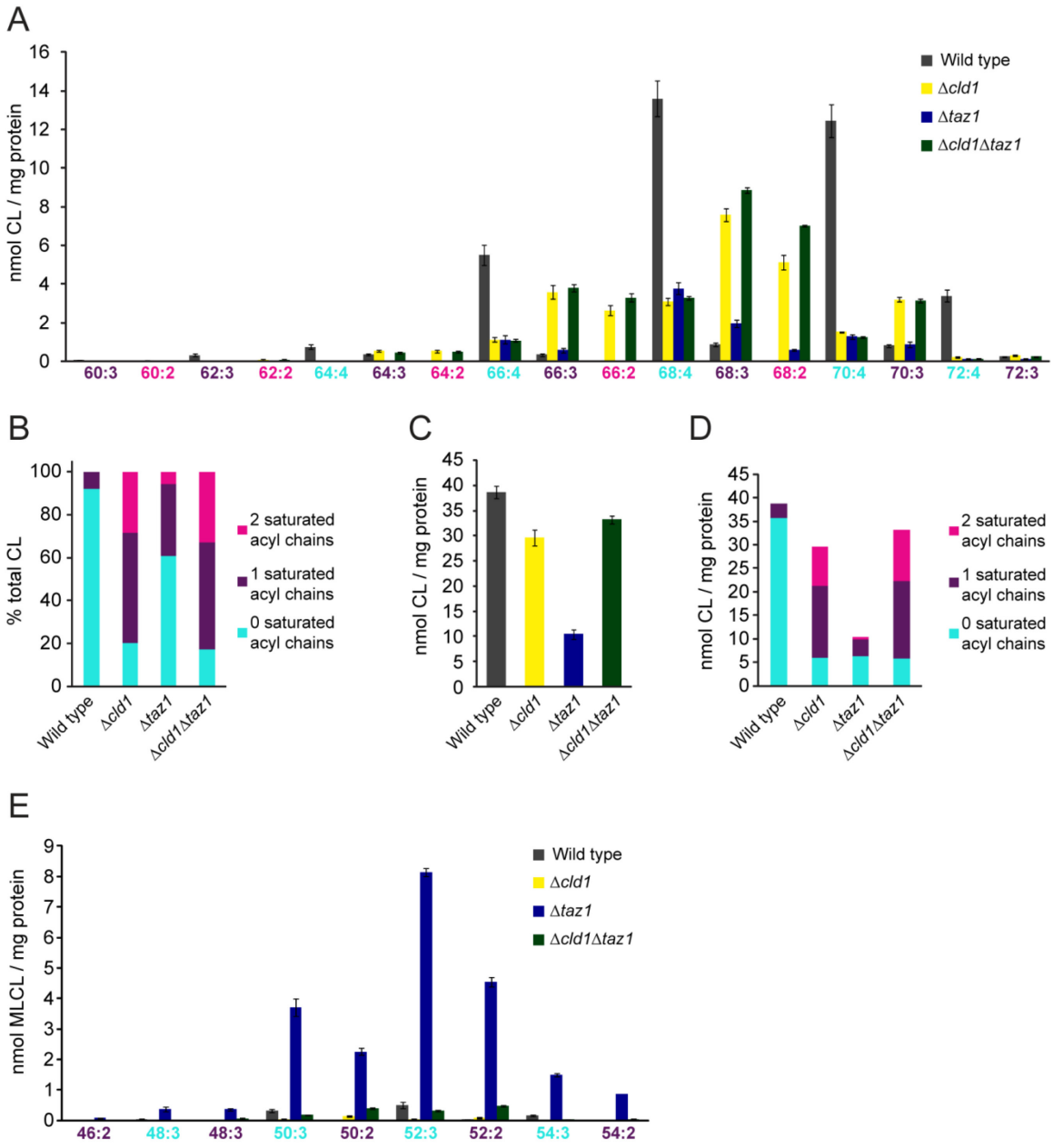
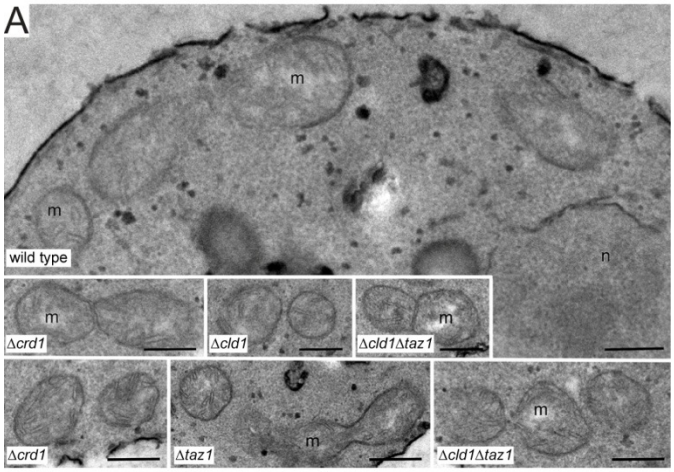
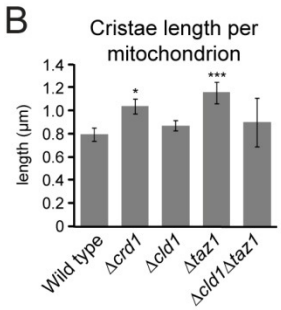
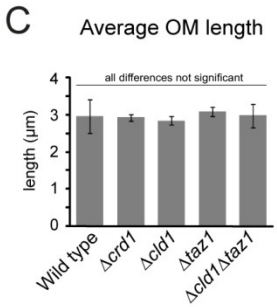

A
nmol CL / mg protein
Wild type
Δcld1
Δtaz1
Δcld1Δtaz1
60:3
60:2
62:3
62:2
64:4
64:3
64:2
66:4
66:3
66:2
68:4
68:3
68:2
70:4
70:3
72:4
72:3
B
% total CL
2 saturated acyl chains
1 saturated acyl chains
0 saturated acyl chains
Wild type
Δcld1
Δtaz1
Δcld1Δtaz1
C
nmol CL / mg protein
D
nmol CL / mg protein
E
nmol MLCL / mg protein
46:2
48:3
48:3
50:3
50:2
52:3
52:2
54:3
54:2

Figure 3.3. Δ*cld1* contains unremodeled CL. (A) The acyl chain composition of CL was determined by multidimensional mass spectrometric array analysis. *n*=3 (B) CL was categorized by the number of saturated acyl chains and expressed as a % of the total CL. (C) Quantification of the total amount of CL per mg of protein. (D) CL was categorized by the number of saturated acyl chains and expressed as the amount of CL per mg protein. (E) The acyl chain composition of MLCL was determined by multidimensional mass spectrometric array analysis. *n*=3. Statistical analysis provided in table S1.







D

	Total Mitochondria	Aberrant Mitochondria	% Aberrant Mitochondria
Wild type	109	4	3.6
Δ*crd1*	124	7	5.6
Δ*cld1*	117	5	4.3
Δ*taz1*	128	9	7.0
Δ*cld1*Δ*taz1*	113	3	2.7

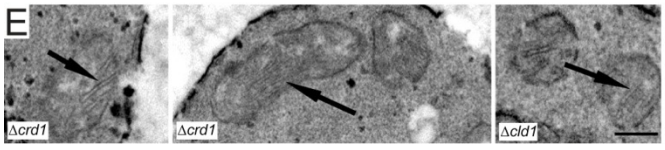
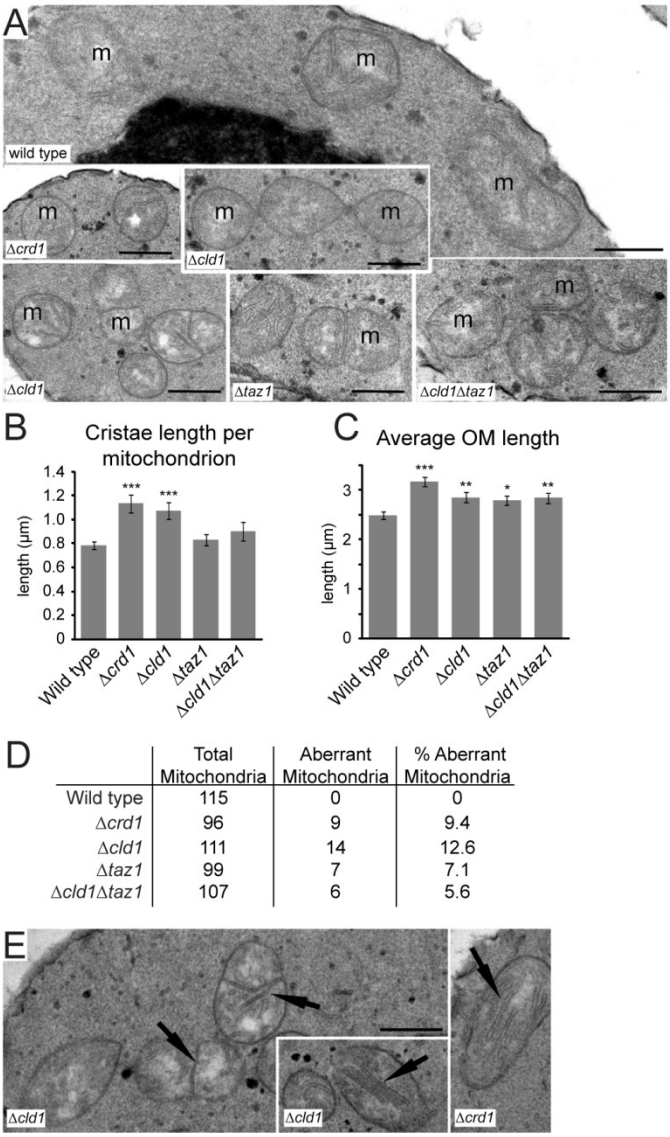


Figure 3.4. Mitochondrial morphology is not affected by unremodeled CL in GA74 yeast. Mitochondria from the indicated strains derived GA74 were analyzed by TEM. (A) Representative micrographs from the indicated strains. m = mitochondria, n = the nucleus. Bars = 0.5 μm. (B) Quantification of cristae length per mitochondrion. (C) Quantification of OM length per mitochondrion. (D) Quantification of aberrant mitochondria for each strain, defined as the appearance of exaggerated cristae >0.5 μm in length. Number of mitochondria analyzed is indicated for each strain. (E) Examples of mitochondria with exaggerated cristae. Bars = 0.5 μm. * $p < 0.05$, *** $p \leq 0.001$.

W303



D

	Total Mitochondria	Aberrant Mitochondria	% Aberrant Mitochondria
Wild type	115	0	0
Δ*crd1*	96	9	9.4
Δ*cld1*	111	14	12.6
Δ*taz1*	99	7	7.1
Δ*cld1*Δ*taz1*	107	6	5.6

Figure 3.5. Mitochondrial morphology is not affected by unremodeled CL in W303 yeast. Mitochondria from the indicated strains derived GA74 were analyzed by TEM. (A) Representative micrographs from the indicated strains. m = mitochondria. Bars = 0.5 μm. (B) Quantification of cristae length per mitochondrion. (C) Quantification of OM length per mitochondrion. (D) Quantification of aberrant mitochondria for each strain, defined as the appearance of exaggerated cristae >0.5 μm in length. Number of mitochondria analyzed is indicated for each strain. (E) Examples of mitochondria with exaggerated cristae. Bars = 0.5 μm. * $p < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

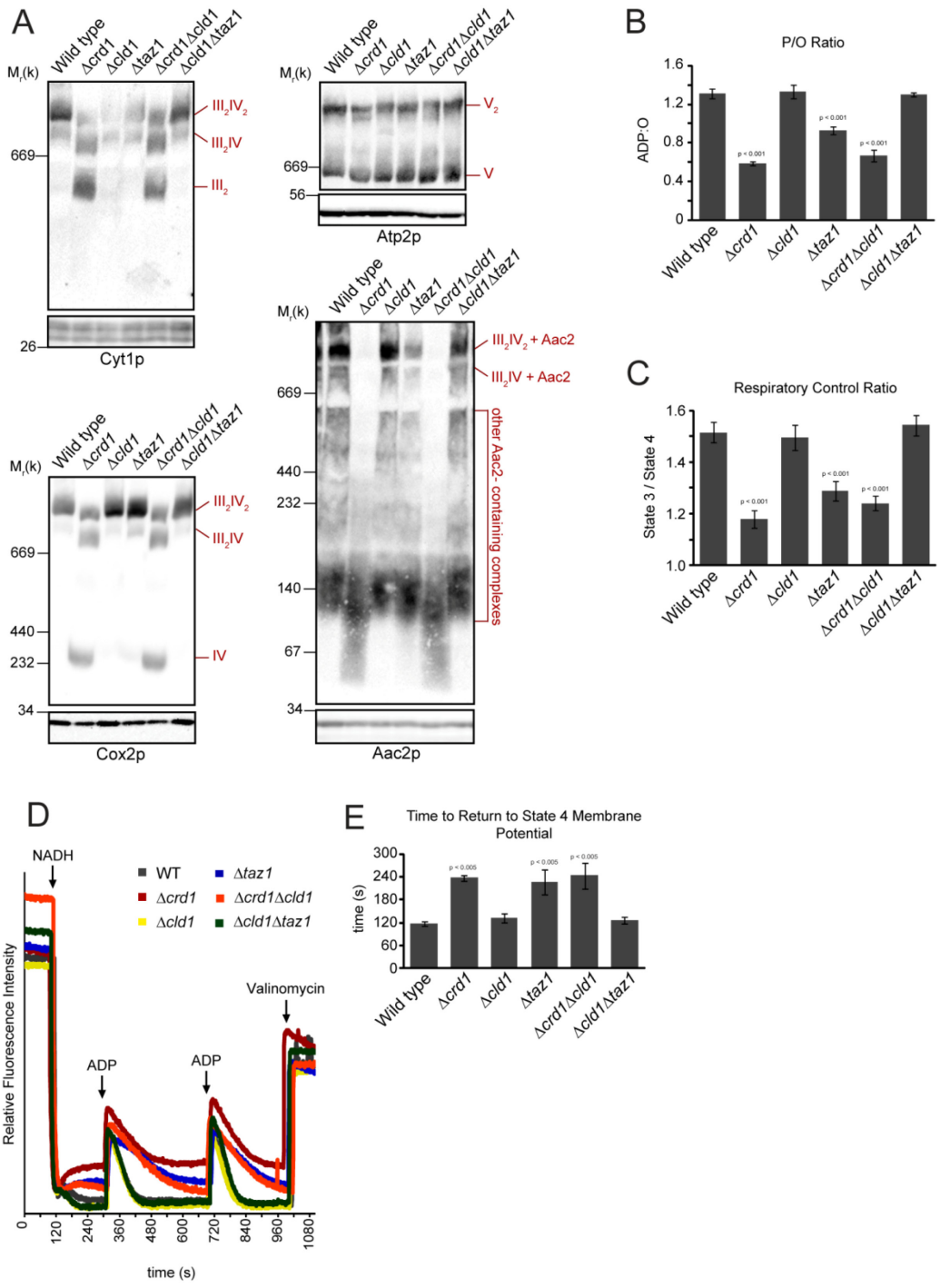

A
Wild type
Δcrd1
Δcld1
Δtaz1
Δcrd1Δcld1
Δcld1Δtaz1
M_r(k)
669
26
III2IV2
III2IV
III2
Cyt1p
669
56
V2
V
Atp2p
669
440
232
34
IV
Cox2p
669
440
232
140
67
34
III2IV2 + Aac2
III2IV + Aac2
other Aac2- containing complexes
Aac2p
B
P/O Ratio
ADP:O
1.6
1.2
0.8
0.4
0
p < 0.001
C
Respiratory Control Ratio
State 3 / State 4
1.6
1.4
1.2
1.0
p < 0.001
D
NADH
WT
Δcrd1
Δcld1
Δtaz1
Δcrd1Δcld1
Δcld1Δtaz1
Valinomycin
ADP
ADP
Relative Fluorescence Intensity
0
120
240
360
480
600
720
840
960
1080
time (s)
E
Time to Return to State 4 Membrane Potential
time (s)
300
240
180
120
60
0
p < 0.005

Figure 3.6. OXPHOS function is not affected by unremodeled CL. (A) Mitochondria were solubilized with digitonin, separated by blue native-PAGE (top panels), and immunoblotted for Cyt1p (complex III), Cox2p (complex IV), Atp2p (complex V), and Aac2p. Bottom panels are immunoblots following SDS-PAGE which serve as loading controls. (B and C) Respiration measured in the presence of 2 mM NADH. n = 6-9. (B) P/O ratios. (C) Respiratory control ratios. (D) Representative TMRM time traces of mitochondria isolated from the indicated yeast strains following the addition of 2 mM NADH (to establish the $\Delta\psi_m$) and two sequential additions of 45 μM ADP to induce phosphorylation cycles, manifest as transient depolarizations. (E) The average times required for the re-establishment of maximal (state 4) $\Delta\psi_m$ following ADP addition for the yeast strains indicated. * $p < 0.05$, *** $p < 0.001$

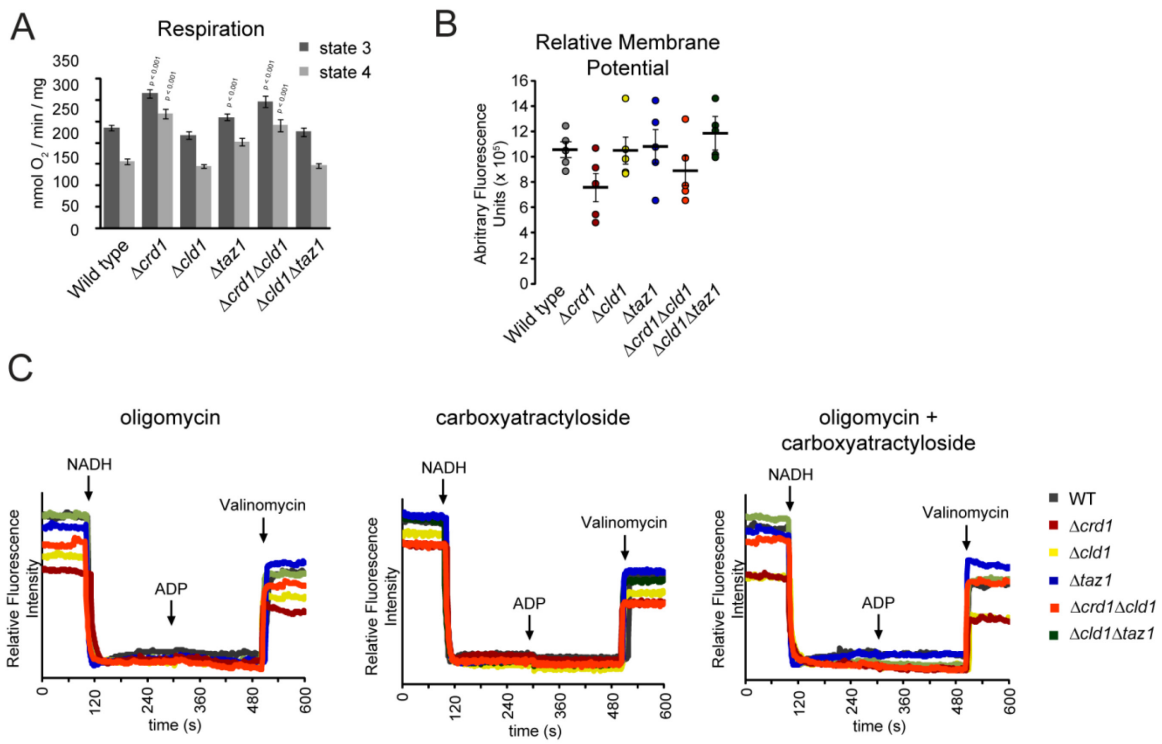


Figure 3.7. Mitochondrial proton leak is increased in the absence of CL. (A) Respiration of isolated mitochondria measured in the presence of NADH (state 4) or NADH and ADP (state 3). *** $p < 0.001$ (B) The relative membrane potentials from 5 independent experiments were plotted (circles). The mean ± SEM are displayed as black bars. (C) Membrane potential of the indicated strains measured in the presence of oligomycin (left), carboxyatractyloside (middle), or oligomycin + carboxyatractyloside (right).

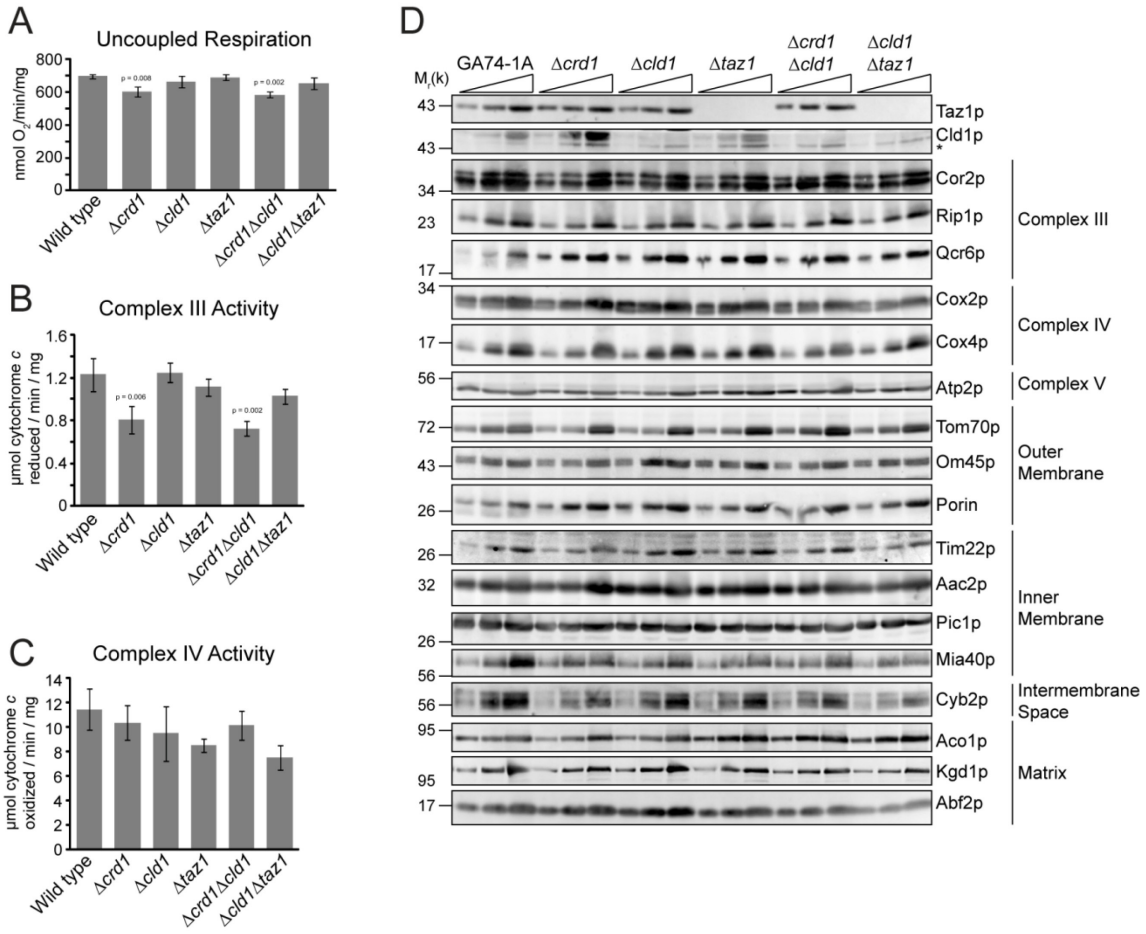


Figure 3.8. The individual components of OXPHOS are not affected by unremodeled CL. (A) Uncoupled respiration measured in the presence of 2 mM NADH and 10 µM CCCP. (B) Complex III and (C) complex IV activity, measured in DDM mitochondrial extracts. ** $p < 0.01$ (D) Mitochondrial proteins from the indicated strains were separated by SDS-PAGE and immunoblotted. * indicates a non-specific cross reaction of the Cld1p antisera

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Chapter 4

Conclusions and unanswered questions

Characterization of Cld1p

In the preceding work, we have characterized the lipase Cld1p, analyzing its topology and regulation in cardiolipin (CL) remodeling, as well as used the Δ*cld1* mutant to analyze the role of specific CL molecular species in supporting mitochondrial function. Using an antibody capable of detecting untagged, endogenous Cld1p, we demonstrated that the lipase resides on the matrix-facing leaflet of the IM and have identified a previously unappreciated trafficking step required for CL remodeling (Figure 4.1). We showed that Cld1p is the major site of regulation for CL remodeling in yeast. Its activity can be regulated by two distinct mechanisms (Figure 4.2): by the available carbon source and by the electrical potential across the IM. These two methods allow CL remodeling to be regulated either coordinately with, or separately from, CL biosynthesis, respectively (chapter 2; Baile et al., 2013b). Additionally, using Δ*cld1*, we showed that unremodeled and remodeled CL are equally able to support OXPHOS and mitochondrial morphology, suggesting that the physiological role of CL remodeling in yeast is not to generate a specific pool of CL molecular species which are optimized for mitochondrial function (chapter 3; Baile et al., 2014). Despite these advances, many questions about CL remodeling remain: How is monolysocardiolipin (MLCL) flipped/transported during CL remodeling? What is the mechanism of CL remodeling regulation? And, what is the physiological role of CL remodeling?

Trafficking in cardiolipin remodeling

Cld1p resides in the matrix-facing leaflet of the IM but does not traverse the membrane. Thus, after Cld1p deacylates CL, MLCL must be trafficked to Taz1p-

containing membranes (Figure 4.1). However, the details of this trafficking remain ill-defined.

No proteins homologous to known flippases/floppases have been identified that are localized to mitochondria (Baile et al., 2013a; Tatsuta et al., 2013). Thus, if the translocation of MLCL is mediated by a flippase or floppase, it would be performed by a class of proteins previously not known to have the capacity to flip phospholipids.

Another possibility is that MLCL flipping is mediated by a phospholipid scramblase. Scramblases catalyze the bidirectional movement of phospholipids between membrane leaflets, generating lipid symmetry in the membrane (Contreras et al., 2010). Potentially, after MLCL is generated by Cld1p in the matrix-facing IM leaflet, MLCL moves down its concentration gradient to the IMS side of the IM, where it is reacylated to CL. The continual deacylation and reacylation of opposite membrane leaflets would maintain MLCL asymmetry across the IM, driving MLCL translocation. However, similar to flippases, mitochondrial phospholipid scramblases are not well defined. Phospholipid scramblase 3 (PLS3), identified in mammals, is the only known scramblase in the mitochondrion (Liu et al., 2003). Unexpectedly, it has been implicated in IM to OM trafficking of CL (Chu et al., 2013; Liu et al., 2003), although it is likely that its CL flipping activity is simply a prerequisite for inter-membrane trafficking (Baile et al., 2013a; Tatsuta et al., 2013). Mutating the putative yeast ortholog of PLS3, *AIM25*, did not affect MLCL translocation (Figure 4.2). The expectation is that in the absence of the protein mediating MLCL translocation, MLCL will accumulate even though Taz1p is functional because MLCL will remain in a membrane leaflet separate from Taz1p. That is not the case in Δ*aim25* yeast. The fact Δ*aim25*Δ*taz1* phenocopies Δ*taz1* further

indicates that Aim25p is not required for the activity/stability of Cld1p. Thus, we conclude that Aim25p does not specifically transport MLCL.

It is possible that Cld1p is inhibited to a certain extent by MLCL, its product. In such a case, the ablation of MLCL flipping would not result in the accumulation of MLCL. Notably, in Δ*taz1* yeast, MLCL would still be able to translocate, preventing the MLCL-mediated inhibition of the flipping protein.

Thus, identifying the protein(s) involved in MLCL translocation will not be trivial. The inability to easily detect MLCL accumulation prevents the use of higher-throughput screening methods (currently the only way to detect MLCL is by extracting mitochondrial lipids and separating them by TLC). The availablility of a biomarker, able to detect MLCL *in vivo*, would greatly facilitate these efforts.

On the other hand, MLCL flipping may not be mediated by a specific protein. Despite asymmetrical phospholipid distributions on ER and bacterial membranes, no proteins mediating phospholipid transbilayer movement have been identified. Instead, it has been suggested that the shear abundance of transmembrane proteins, rather than specific proteins, facilitate transbilayer movement of phospholipids (Kol et al., 2004; Kol et al., 2001; van Meer et al., 2008). The high protein:lipid ratio in the IM (Hallermayer and Neupert, 1974; Simbeni et al., 1991) would certainly make it a candidate for such a mechanism.

Compared to its four hydrophobic acyl chains, CL has a relatively small polar headgroup. Thus, it is conceivable that flipping is not mediated by proteins at all, but rather via biophysical mechanisms, similar to the way in which PA can flip between

membrane leaflets in response to changes in pH (Gallet et al., 1999; Hope et al., 1989). However, this hasn't been formally demonstrated for either CL or MLCL.

That Taz1p also resides on the OM (Claypool et al., 2006; Gebert et al., 2009) suggests that MLCL can also be trafficked between the IM and OM. Although proteins mediating CL trafficking between membranes have been identified (Epand et al., 2007; Liu et al., 2003; Schlattner et al., 2013), whether they can also transport MLCL or if different players are involved remains an unanswered question.

Regulating cardiolipin remodeling

CL remodeling can be regulated by two separate mechanisms (Figure 4.2; chapter 2; Baile et al., 2013b). In the first, the presence of non-fermentable carbon sources promotes Cld1p activity (and thus remodeling) by increasing its expression. Consistent with this, a recent report suggests that *CLD1* transcription is activated by the Hap2p/3p/4p/5p and Mig1p transcription factors (Ye et al., 2013), which regulate gene expression in response to glucose (DeRisi et al., 1997; Nehlin and Ronne, 1990; Schuller, 2003).

Increasing the activity of CL remodeling in the presence of non-fermentable carbon sources is intuitive; these conditions promote mitochondrial biogenesis and result in increased CL abundance, and thus it is not surprising that CL remodeling would also be increased. However, it remains unknown whether the increase in CL remodeling activity is designed to keep pace with the increased synthesis of CL, or if the increase in CL remodeling also results in a larger proportion of remodeled CL. We have shown that in rich lactate media, only ~8% of CL contains a saturated acyl chain (chapter 3; Baile et

al., 2014), but a direct comparison of the CL acyl chain composition from yeast grown in dextrose *vs.* lactate has not been performed.

The second identified mechanism of CL remodeling regulation, the dissipation of the mitochondrial membrane potential, is less straightforward. Dissipation of the electrochemical gradient (both the membrane potential and the pH gradient across the IM) has opposite effects on CL biosynthesis and remodeling. A decrease in the pH gradient reduces the activity of Crd1p (Gohil et al., 2004), but a decrease in the membrane potential stimulates Cld1p (chapter 2; Baile et al., 2013b). Thus, in principle, CL biosynthesis and remodeling could be independently regulated. However, it is unclear if physiological conditions exist where the matrix pH is affected without changing the membrane potential, and vice versa. Such a mechanism would require the movement (or countermovement) of ions across the IM independent from the movement of protons.

Mechanistically, it is unknown how dissipation of the membrane potential can affect the activity of Cld1p. Since the membrane potential is generated by pumping protons (positive charges) out of the matrix (the location of Cld1p) and into the IMS, dissipation of the membrane potential essentially results in an increase of positively charged ions in the matrix. Whether this directly affects CL binding to Cld1p or the catalytic activity of Cld1p, or instead serves as a signal which leads to a post translational and/or allosteric modification of Cld1p remains to be investigated, and will likely require the reconstitution of Cld1p activity *in vitro*.

Decreases in the membrane potential can occur as the result of decreased proton pumping by the electron transport chain. This may be due to either dysfunctional

OXPHOS (either by mutation or chemical insult), or during low energy charge caused by either a lack of substrates providing reducing equivalents to the electron transport chain or the increased cellular use of ATP. Although this provides a link between CL remodeling and OXPHOS capacity, the physiological outcome of remodeling CL remains enigmatic.

The physiological role of cardiolipin remodeling

As discussed previously (chapter 3; Baile et al., 2014), the physiological role of CL remodeling remains an unanswered question. The prevailing hypothesis that CL remodeling establishes a specific molecular form of CL that is better able to support mitochondrial function was shown to be incorrect, at least in terms of OXPHOS function and mitochondrial morphology in yeast under optimal conditions. It is worth mentioning that, although we tested only OXPHOS capacity and mitochondrial morphology, other processes requiring CL would indirectly affect these two parameters. Protein import defects are expected to reduce the steady state abundance of nuclear encoded respiratory complex subunits causing OXPHOS defects (Kulawiak et al., 2013), and defects in mitochondrial fission or fusion cause OXPHOS deficiency as well as alter mitochondrial morphology (for examples, see Amiott et al., 2009; Amiott et al., 2008; Amutha et al., 2004; Bleazard et al., 1999; Chen et al., 2005; Hoppins et al., 2009; Koshiba et al., 2004; Meeusen et al., 2006; Otsuga et al., 1998; Sesaki et al., 2003). That we saw no OXPHOS or morphology defects in Δ*cld1* suggests (but does not unequivocally show) that protein import and mitochondrial dynamics are also not affected.

In an effort to uncover the physiological role of CL remodeling, we searched for genes that genetically interact with *CLD1*, focusing on those known to genetically

interact with other genes in the CL biosynthesis and remodeling pathway. *PSD1*, which encodes the mitochondrial phosphatidylserine (PS) decarboxylase, converts PS to phosphatidylethanolamine (PE) (Clancey et al., 1993; Zinser et al., 1991). In the absence of CL, mitochondrially produced PE is absolutely required as demonstrated by the synthetic lethality of Δ*crd1*Δ*psd1* yeast (Gohil et al., 2005). No growth defect was observed in Δ*cld1*Δ*psd1* yeast (Figure 4.4A), suggesting that the synthetic lethality of Δ*crd1*Δ*psd1* is due to the absence of total CL, not remodeled CL, in the absence of PE.

CRD1 also displays synthetic growth defects with components of the translocase of the outer membrane (TOM) complex, which may indicate a specific role of CL in the assembly or stability of the TOM complex (Gebert et al., 2009). Tom5p is important for the transfer of protein precursors from the TOM receptor to the import pore (Dietmeier et al., 1997) and the assembly and stability of the TOM complex (Becker et al., 2010; Schmitt et al., 2005). Deletion of *TOM5* in Δ*crd1* and Δ*taz1* yeast resulted in synthetic growth defects (Figure 4.4B), as expected (Gebert et al., 2009), although in this genetic background Δ*taz1*Δ*tom5* was still viable at 30°C. However, there was no synthetic growth defect in the Δ*cld1*Δ*tom5* strain. Likewise, deletion of *TOM70*, which encodes a receptor of the TOM complex (Hines et al., 1990), in either Δ*crd1* or Δ*taz1* yeast results in a growth defect at either elevated temperature or on respiratory media, but no growth defect is observed in the Δ*cld1*Δ*tom70* strain (Figure 4.4C). Thus, the synthetic growth defects observed between yeast with no (Δ*crd1*) or reduced (Δ*taz1*) CL and TOM complex mutants is due to the changes in CL abundance and not CL acyl chain composition.

UPS proteins regulate the levels of CL and PE (Osman et al., 2009; Tamura et al., 2009). Ups1p transports PA from the OM to the IM upstream of Tam41p in CL biosynthesis (Connerth et al., 2012), and Ups2p antagonizes Ups1p (Tamura et al., 2009) and regulates mitochondrial PE levels through currently ill-defined mechanisms (Tamura et al., 2012a; Tamura et al., 2012b). *UPS1* and *UPS2* both have been shown to display synthetic growth defects with *CRD1* and *TAZ1* (Costanzo et al., 2010; Hoppins et al., 2011; Osman et al., 2009), consistent with our results (Figure 4.4D and E), but neither the Δ*cld1*Δ*ups1* nor the Δ*cld1*Δ*ups2* strain displayed any sort of synthetic growth defect.

There appeared to be a positive genetic interaction between *CLD1* and *MDM10*, an OM protein which participates in both the ER-mitochondria encounter structure (ERMES) complex (Kornmann et al., 2009) and the sorting and assembly machinery (SAM) complex (Yamano et al., 2010) (Figure 4.5A), but that proved to be strain dependent, resulting in a negative genetic interaction when the same strain was constructed in a different genetic background (Figure 4.5B). Notably, Δ*mdm10* yeast rapidly lose their mitochondrial genome (Hanekamp et al., 2002; our unpublished results). Thus, all strain containing wild type *MDM10* were also ρ^-.

Our search for genes that interact with *CLD1* yielded only negative results. However, the genes selected were limited, and biased toward genes already known to interact with other members of CL biosynthesis or remodeling. An alternative, and potentially better, approach would be a non-biased, genome-wide screen for genetic interactions with *CLD1*. This may need to be combined with the addition of different stressors (chemicals, temperatures, carbon sources, etc) to reveal a phenotype.

While no synthetic growth defect was observed between *CLD1* and *UPS1*, analysis of the phospholipids shows a greater reduction of CL in both the Δ*cld1*Δ*ups1* and Δ*taz1*Δ*ups1* strains than any of the single mutants alone (Figure 4.6). Additionally, no MLCL accumulated in the Δ*taz1*Δ*ups1* mutant. Taken together, these data suggest that in the absence of *UPS1* and a functional CL remodeling pathway, CL biosynthesis is decreased. Further studies are required to reveal the mechanism, and the functional relevance, of this potential mode of regulation.

Our difficulty in identifying functional differences between remodeled and unremodeled CL may be a result of Δ*cld1* being an imperfect (albeit the best currently available) model for unremodeled CL. It is possible that the ~20% of CL in Δ*cld1* containing four unsaturated acyl chains (and thus, looks like remodeled CL) is sufficient to maintain mitochondrial function. To circumvent this issue, the abundance of CL could be modulated (perhaps by expressing a CL biosynthetic enzyme under the control of an inducible promoter). If the remaining remodeled CL in Δ*cld1* were able to support mitochondrial function, then this strain should be more sensitive to decreases in CL abundance than a strain able to perform CL remodeling.

Potentially, the establishment of specific CL molecular species is more important for metazoans than for yeast, although this is hard to imagine since the Taz1p-mediated remodeling pathway is conserved between yeast and metazoans. A direct test of this would first require that the lipase(s) initiating CL remodeling are identified in metazoans.

Remodeling the model of cardiolipin remodeling

If not to establish a specific molecular form of CL, what then is the physiological purpose of CL remodeling? One possibility is that CL remodeling is required for CL

trafficking. We have demonstrated that in order to gain access to Taz1p-containing membranes and complete remodeling, MLCL must either flip to the IMS-facing leaflet of the IM or to the OM (chapter 2; Baile et al., 2013b). Deacylation of CL to MLCL may be a critical step in trafficking. Although other proteins have been implicated in CL trafficking (Epand et al., 2007; Liu et al., 2003; Schlattner et al., 2013), their roles remain unclear, especially considering that clear orthologs in yeast have yet to be established (see discussion in chapter 1). Additionally, NDPK-D and MtCK have been suggested to transfer CL as a step in apoptosis (Epand et al., 2007). Thus, how CL translocates to the OM during normal physiological conditions remains unclear.

A second possibility, which is not mutually exclusive with CL remodeling being required for MLCL transport, is that CL remodeling could serve as a repair mechanism. We showed that CL remodeling activity increases when the membrane potential dissipates. Mitochondria are a major source of reactive oxygen species (ROS) in cells. Since CL is closely associated with electron transport chain complexes, it is highly susceptible to ROS-mediated damage. CL with oxidized acyl chains inhibits the function of some OXPHOS complexes. Thus, when ROS damages CL, protons are pumped less efficiently and the membrane potential decreases, activating CL remodeling. Acyl chains are removed from CL and replaced with new acyl chains, restoring OXPHOS function. Importantly, our analysis of OXPHOS and mitochondrial morphology were performed under optimal conditions, which may not result in substantial ROS levels. Although increased oxidative damage was observed in Δ*taz1* yeast, it was only observed when the yeast were grown at elevated temperature (Chen et al., 2008). Thus, analysis of Δ*cld1* mitochondria from yeast grown at elevated temperature, or after inducing ROS

production (either chemically or genetically), may reveal differences between unremodeled and remodeled CL.

Analysis of CL molecular species in the Δ*taz1* suggests that Cld1p preferentially removes saturated acyl chains. Unsaturated fatty acyl chains have a different shape than saturated acyl chains; the double bond between carbon atoms in unsaturated acyl chains induces a "kink" in its molecular structure. Intriguingly, peroxidation of an unsaturated acyl chain causes it to lose its double bond, returning it to a straight chain. Therefore, it is possible that the substrate specificity of Cld1 is provided by the shape of the acyl chain, although this has never been formally demonstrated.

If CL remodeling serves as a CL repair mechanism, what are the advantages of generating CL with unsaturated acyl chains, which are highly susceptible to oxidative damage? One possibility is that by placing remodeled CL in close proximity to the ROS-producing respiratory complexes, it can act as a buffer to absorb ROS-mediated damage. CL has a dedicated pathway to remove and replace acyl chains; other mitochondrial components do not. Thus, we speculate that CL can limit oxidative damage to mitochondrial proteins and the mitochondrial genome by being the initial target of oxidative damage.

MATERIALS AND METHODS

All yeast strains used in this study were isogenic to GA74-1A and described in table 4.1, except those in figure 4.5B which are isogenic to PTY144 (Thorsness et al., 2002) and are described in table 4.2. Genetic knockouts were generated by replacing the entire open reading frame via homologous recombination as described previously (Wach et al., 1994). Double mutants were generated by homologous recombination or by mating and sporulation, as indicated in table 4.1. ρ^- strains were generated by plating yeast on YPD (1% yeast extract, 2% peptone, 2% dextrose) + 40 μg/ml ethidium bromide twice consecutively. ρ^- strains were verified by the absence of Cox2p expression via immunoblot. During strain construction, Δ*crd1*Δ*tom5,* Δ*crd1*Δ*ups2* and Δ*crd1*Δ*mdm10* were covered with the plasmid pRS426*CNAPmCRD1* (*CRD1* with a CNAP tag (Claypool et al., 2008b) appended after amino acid 55), Δ*cld1*Δ*tom5* and Δ*cld1*Δ*mdm10* were covered with the plasmid pRS426*CLD1*, and Δ*taz1*Δ*tom5* was covered with the plasmid pRS426*TAZ1*, and the plasmid was selected against by plating twice consecutively on synthetic media (0.17% yeast nitrogen base, 0.5% ammonium sulfate, 0.2% complete amino acid mix) containing 2% (w/v) dextrose and 1 mg/ml 5-fluoroorotic acid. Absence of the plasmid was verified by immunoblot. Growth analysis was performed by spotting 3 μl of overnight cultures grown in YPD or YPD + 1 M sorbitol (for analysis of strains containing Δ*ups1* or Δ*ups2*) and spotted on synthetic media with either 2% (w/v) dextrose or 3% (v/v) glycerol/1% (v/v) ethanol as the sole carbon source. Phospholipid analysis was performed as described previously (Claypool et al., 2006).

Table 4.1. Yeast strains used derived from GA74

Strain	Genotype	Parental strain	Reference/ source
GA74-1A	*MAT***a**, *his3-11,15*, *leu2*, *ura3*, *trp1*, *ade8* [ρ^+, *mit*$^+$]		
Δ*crd1*	*MAT***a**, *his3-11,15, leu2, ura3, ade8,* Δ*crd1*::*TRP1*	GA74-1A	(Claypool et al., 2008b)
Δ*cld1*	*MAT***a**, *leu2, ura3, trp1, ade8,* Δ*cld1*::*HISMX6*	GA74-1A	(Baile et al., 2013b)
Δ*taz1*	*MAT***a**, *leu2, ura3, trp1, ade8,* Δ*taz1*::*HIS3MX*	GA74-1A	(Claypool et al., 2008a)
Δ*taz1*::U	*MAT***a**, *his3-11,15, leu2, trp1, ade8,* Δ*taz1*::*URA3MX*	GA74-1A	(Baile et al., 2013b)
Δ*psd1*	*MAT***a**, *his3-11,15, leu2, ura3, ade8,* Δ*psd1*::*TRP1*	GA74-1A	This study
Δ*cld1*Δ*psd1*	*MAT***a**, *leu2, ura3, ade8,* Δ*psd1*::*TRP1,* Δ*cld1*::*HISMX6*	Δ*psd1*	This study
Δ*tom5*	*MAT***a**, *leu2, ura3, trp1, ade8,* Δ*tom5*::*HISMX6*	GA74-1A	This study
Δ*crd1*Δ*tom5*	*MAT***a**, *leu2, ura3, ade8,* Δ*crd1*::*TRP1,* Δ*tom5*::*HISMX6*	Δ*crd1*	This study
Δ*cld1*Δ*tom5*	*MAT***a**, *leu2, ura3, ade8,* Δ*cld1*::*HISMX6,* Δ*tom5*::*TRP1*	Δ*cld1*	This study
Δ*taz1*Δ*tom5*	*MAT***a**, *leu2, ura3, ade8,* Δ*taz1*::*HISMX6,* Δ*tom5*::*TRP1*	Δ*taz1*	This study
Δ*tom70*	*MAT***a**, *his3-11,15*, *leu2*, *trp1*, *ade8,* Δ*tom70*::*URA3MX*	GA74-1A	This study
Δ*crd1*Δ*tom70*	*MAT***a**, *his3-11,15*, *leu2*, *ade8,* Δ*crd1*::*TRP1* Δ*tom70*::*URA3MX*	Δ*crd1*	This study
Δ*cld1*Δ*tom70*	*MAT***a**, *leu2*, *trp1*, *ade8,* Δ*cld1*::*HISMX6,* Δ*tom70*::*URA3MX*	Δ*cld1*	This study
Δ*taz1*Δ*tom70*	*MAT***a**, *his3-11,15*, *leu2*, *ade8,* Δ*taz1*::*URA3MX,* Δ*tom70*::*TRP1*	Δ*taz1*::U	This study
Δ*ups1*	*MAT***a**, *leu2, ura3, trp1, ade8,* Δ*ups1*::*HISMX6*	GA74-1A	This study
Δ*crd1*Δ*ups1*	*MAT***a**, *leu2, ura3, ade8,* Δ*crd1*::*HIS3MX6,* Δ*ups1*::*TRP1*	Δ*crd1*/*CRD1*, Δ*ups1*/*UPS1*	This study
Δ*cld1*Δ*ups1*	*MAT***a**, *leu2, ura3, ade8,* Δ*cld1*::*HISMX6,* Δ*ups1*::*TRP1*	Δ*cld1*	This study
Δ*taz1*Δ*ups1*	*MAT***a**, *his3-11,15*, *leu2*, *ade8,* Δ*taz1*::*URA3MX,* Δ*ups1*::*TRP1*	Δ*taz1*/*TAZ1*, Δ*ups1*/*UPS1*	This study
Δ*ups2*	*MAT***a**, *his3-11,15, leu2, ura3, ade8,* Δ*ups2*::*TRP1*	GA74-1A	This study
Δ*crd1*Δ*ups2*	*MAT***a**, *leu2, ura3, ade8,* Δ*crd1*::*TRP1,* Δ*ups2*::*HIS3MX6*	Δ*crd1*	This study
Δ*cld1*Δ*ups2*	*MAT***a**, *leu2, ura3, ade8,* Δ*cld1*::*HIS3MX6,* Δ*ups2*::*TRP1*	Δ*cld1*	This study
Δ*taz1*Δ*ups2*	*MAT***a**, *leu2, ura3, ade8,* Δ*cld1*::*HIS3MX6,* Δ*ups2*::*TRP1*	Δ*ups2*	This study

Table 4.1 (continued). Yeast strains used derived from GA74

Strain	Genotype	Parental strain	Reference/ source
Δ*mdm10*	*MAT***a**, *leu2*, *ura3*, *trp1*, *ade8*, Δ*mdm10*::*HIS3MX6* [ρ^-]	GA74-1A	This study
Δ*crd1*Δ*mdm10*	*MAT***a**, *leu2*, *ura3*, *ade8*, Δ*crd1*::*TRP1*, Δ*mdm10*::*HIS3MX6* [ρ^-]	Δ*crd1*	This study
Δ*cld1*Δ*mdm10*	*MAT***a**, *leu2*, *ura3*, *ade8*, Δ*cld1*::*HIS3MX6*, Δ*mdm10*::*TRP1* [ρ^-]	Δ*cld1*	This study
Δ*taz1*Δ*mdm10*	*MAT***a**, *leu2*, *trp1*, *ade8*,Δ*taz1*::*HIS3MX6*, Δ*mdm10*::*URA3MX* [ρ^-]	Δ*taz1*/*TAZ1*, Δ*mdm10/MDM10*	This study
GA74-1A ρ^-	*MAT***a**, *his3-11,15*, *leu2*, *ura3*, *trp1*, *ade8* [ρ^-]	GA74-1A	This study
Δ*crd1* ρ^-	*MAT***a**, *his3-11,15, leu2, ura3, ade8*, Δ*crd1*::*TRP1* [ρ^-]	Δ*crd1*	
Δ*cld1* ρ^-	*MAT***a**, *leu2, ura3, trp1, ade8*, Δ*cld1*::*HISMX6* [ρ^-]	Δ*cld1*	This study
Δ*taz1* ρ^-	*MAT***a**, *leu2, ura3, trp1, ade8*, Δ*taz1*::*HIS3MX* [ρ^-]	Δ*taz1*	

Table 4.2. Yeast strains used derived from PTY144

Strain	Genotype	Parental strain	Reference/ source
PTY144	*MAT*α, *leu2-3,112, ura3-52, trp1-*Δ*1, lys2, his3::hisg* [ρ^+, *LYS2*]	D273	(Thorsness et al., 2002)
PTY144 ρ^-	*MAT*α, *leu2-3,112, ura3-52, trp1-*Δ*1, lys2, his3::hisg* [ρ^-, *LYS2*]	PTY144	This study
Δ*cld1*	*MAT*α, *leu2-3,112, ura3-52, trp1-*Δ*1, lys2,* Δ*cld1*::*HIS3MX6* [ρ^+, *LYS2*]	PTY144	(Baile et al., 2014)
Δ*cld1* ρ^-	*MAT*α, *leu2-3,112, ura3-52, trp1-*Δ*1, lys2,* Δ*cld1*::*HIS3MX6* [ρ^-, *LYS2*]	Δ*cld1*	This study
Δ*mdm10*	*MAT*α, *leu2-3,112, trp1-*Δ*1, lys2, his3::hisg,* Δ*mdm10*::*URA3MX* [ρ^-, *LYS2*]	PTY144	This study
Δ*cld1*Δ*mdm10*	*MAT*α, *leu2-3,112, trp1-*Δ*1, lys2,* Δ*cld1*::*HIS3MX6,* Δ*mdm10*::*URA3MX* [ρ^-, *LYS2*]	Δ*cld1*	This study

FIGURES

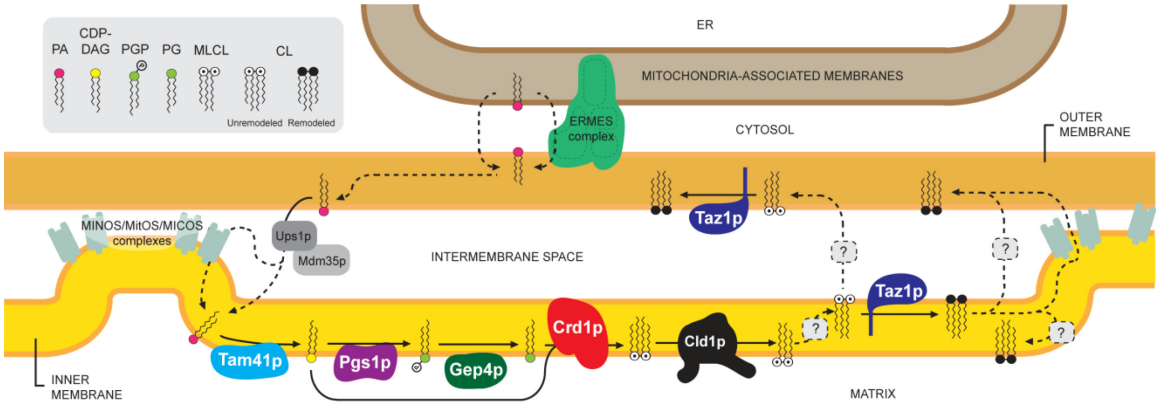

Figure 4.1. The topology of CL biosynthesis and remodeling. Phosphatidic acid (PA) is synthesized in the ER and translocates to mitochondria in a process that is influenced by the ERMES (ER-mitochondria encounter structure) complex. Ups1/Mdm35p heterodimers transport PA from the OM to the IM, potentially at contact sites (established by MINOS/MICOS/MitOS complexes). PA is converted to CDP-diacylglycerol (CDP-DAG) by Tam41p on the matrix-facing leaflet of the IM. CDP-DAG is used to generate phosphatidylglycerolphosphate (PGP) by Pgs1p. PGP is dephosphorylated to phosphatidylglycerol (PG) by Gep4p. PG and another CDP-DAG are condensed to form unremodeled CL by Crd1p. CL is deacylated by Cld1p on the matrix-facing leaflet of the IM, forming MLCL. Via an unknown mechanism, MLCL must flip to the IMS-facing leaflet of the IM or be transported to the OM to gain access to the transacylase Taz1p, which regenerates CL. Multiple rounds of deacylation/reacylation result in remodeled CL which is enriched in unsaturated acyl chains. CL achieves its final distribution on both leaflets of the IM and OM through currently ill-defined mechanisms. The depicted topology of Pgs1p has not been experimentally verified. Solid lines indicate known pathways. Dashed lines delineate potential but currently unknown phospholipid transport processes.

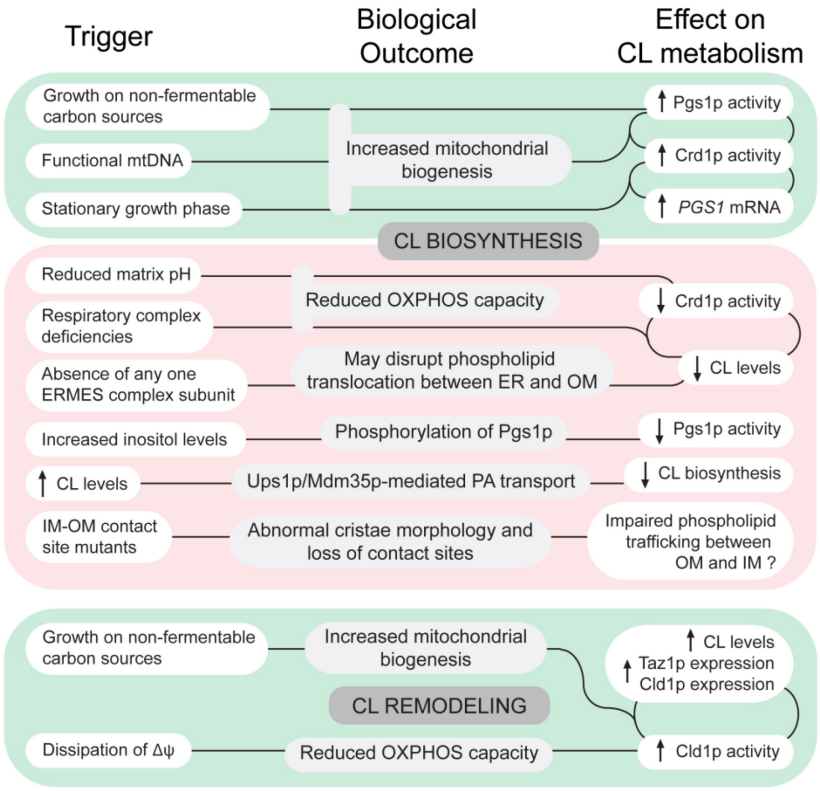

Figure 4.2. Regulatory mechanisms of CL biosynthesis and remodeling. The CL biosynthetic pathway is upregulated under conditions favoring mitochondrial biogenesis. In contrast, deficiencies in ERMES (ER-OM), MINOS/MitOS/MICOS (OM-IM contact sites) complexes, and components of the electron transport chain, as well as increased levels of inositol and reduced matrix pH, can all lead to a down-regulation of CL biosynthesis. Additionally, CL levels can be modulated by Ups1p/Mdm35p-mediated PA transport. Similar to CL biosynthesis, growth of yeast on respiratory media also promotes CL remodeling by upregulating the activity and/or expression of enzymes in the remodeling pathway. Distinct from CL biosynthesis, dissipation of the electrical potential across the IM, indicative of reduced OXPHOS capacity, increases Cld1p activity. Green boxes indicates conditions that promote CL biosynthesis and remodeling while red boxes indicate conditions that repress CL biosynthesis.

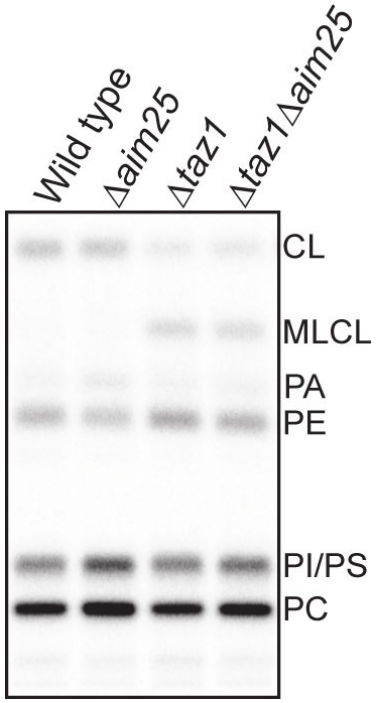

Figure 4.3. *AIM25* is not required for MLCL redistribution. Mitochondrial phospholipids from yeast grown in the indicated media were labeled with $^{32}P_i$ and separated by TLC.

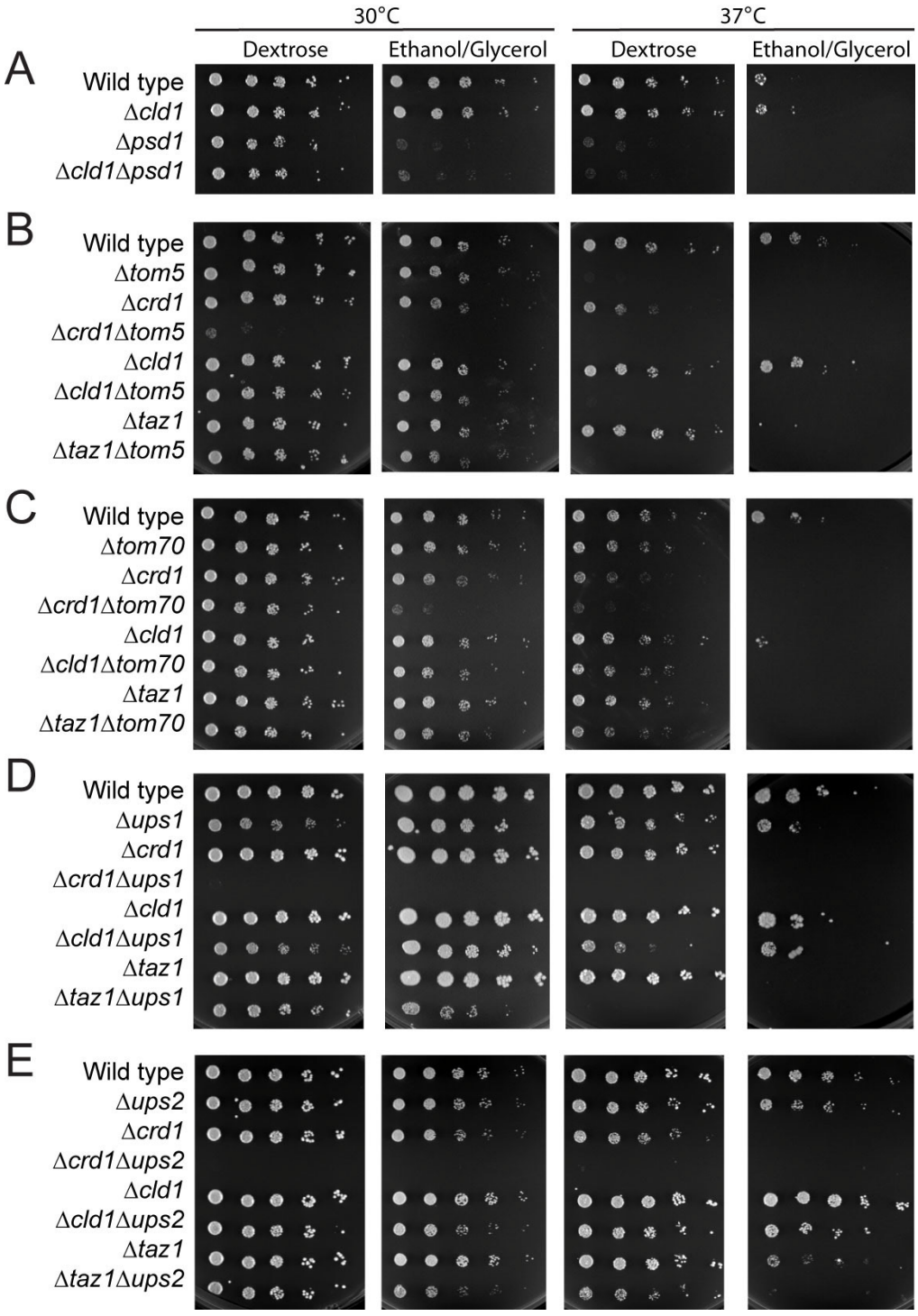

Figure 4.4. **Genetic interactions with CL biosynthesis and remodeling pathways.** 1:4 serial dilutions of the indicated yeast strain were spotted onto synthetic media containing dextrose or ethanol/glycerol as the sole carbon source, and incubated at 30°C or 37°C.

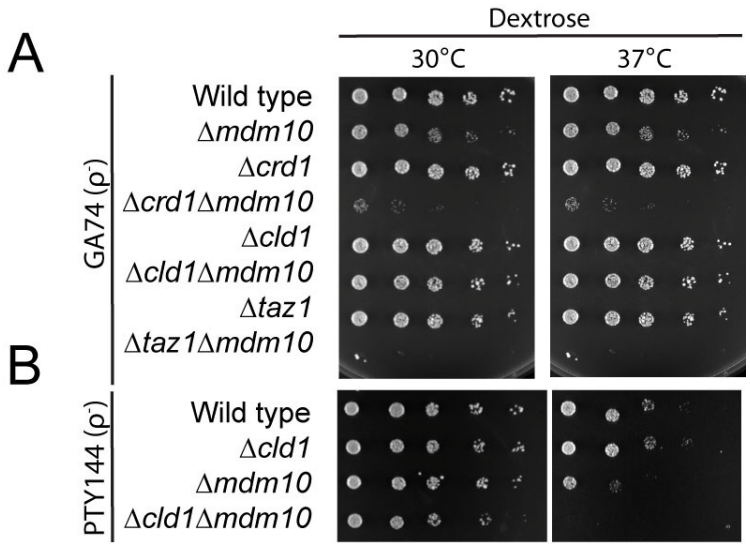

Figure 4.5. Strain dependent genetic interactions between *CLD1* and *MDM10*. 1:4 serial dilutions of the indicated yeast strain derived from (A) GA74 ρ^- or (B) PTY144 ρ^- were spotted onto synthetic media containing dextrose as the sole carbon source and incubated at 30°C or 37°C.

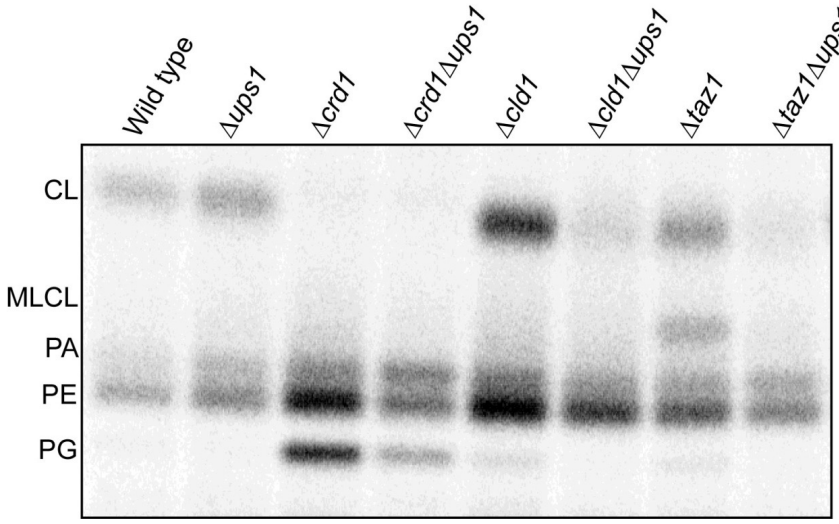

Figure 4.6. CL biosynthesis is decreased in the absence of *UPS1* and functional CL remodeling. Mitochondrial phospholipids from the indicated strains were labeled with $^{32}P_i$ and separated by TLC.

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Publications:

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